Vol. XIV, No. 2 April 1920

THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

W. M. BAYLISS, F.R.S.

AND

ARTHUR HARDEN, F.R.S.

EDITORIAL COMMITTEE

Dr G. BARGER
Prof. V. H. BLACKMAN
Mr J. A. GARDNER
Prof. F. G. HOPKINS
Prof. F. KEEBLE
Prof. B. MOORE
Prof. W. RAMSDEN
Dr E. J. RUSSELL

CAMBRIDGE UNIVERSITY PRESS
C. F. Clay, Manager
London: Fetter Lane, E.C. 4
also
H. K. Lewis & Co., Ltd., 136, Gower Street, London, W.C. 1
William Wesley & Son, 28, Essex Street, Strand, London, W.C. 2
Chicago: The University of Chicago Press
Bombay, Calcutta, Madras: Macmillan and Co., Ltd.
Toronto: The Macmillan Co. of Canada, Ltd.
Tokyo: The Maruzen-Kabushiki-Kaisha

1920

Price Fourteen Shillings net

The *Biochemical Journal* is conducted by the Biochemical Society and is published by the Cambridge University Press.

This Society has been instituted for the purpose of facilitating intercourse between those biologists and chemists who are interested in the investigation of problems common to both, such as the chemical problems connected with Agriculture, Brewing, Animal and Vegetable Physiology and Pathology, &c. Persons interested in Biochemistry are eligible for election.

Meetings are held at different centres for the communication of papers to the Society.

The annual subscription is 35*s.*, which includes a copy of the Journal, and becomes due on January 1st. Further information may be obtained on application to the Hon. Sec., Dr J. C. Drummond, University College, London, W.C. 1, to whom subscriptions should be sent.

Papers for publication should be sent to Prof. W. M. Bayliss, F.R.S., University College, W.C. 1, or to Prof. A. Harden, F.R.S., Lister Institute, Chelsea Gardens, S.W. 1. Communications respecting the printing of the articles, or respecting the purchase of offprints should be addressed to the University Press, Cambridge.

The Journal is issued about every two months and the date at which each paper is received by the editors is printed at the beginning of the paper.

All communications respecting the purchase of copies of the parts or volumes, whether current or back issues, or respecting subscriptions in the case of non-members of the Biochemical Society (£2. 2*s.* net per volume (post free) payable in advance) should be addressed to Mr C. F. Clay, Cambridge University Press, Fetter Lane, London, E.C. 4. For prices of back numbers and volumes see p. 4 of this wrapper.

Quotations can be given for Buckram binding cases and for binding Subscribers' Sets.

The Cambridge University Press has appointed the University of Chicago Press agents for the sale of the *Biochemical Journal* in the United States of America and has authorised them to fix the following subscription price. $10.50 net.

Notice to Contributors.

1. Contributors receive 50 copies of their papers free and can purchase additional copies if notice be given when their proofs are returned.

2. Papers forwarded to the Editors for publication are understood not to be offered to any other Journal for prior or simultaneous publication.

3. All communications intended for publication must be type-written.

4. **References.** References to literature should be arranged alphabetically according to authors' names at the end of the communication, each accompanied by the date, title of Journal, volume and page, thus: Ackermann (1912, 1), *Zeitsch. Biol.* 59, 17. In the text the reference should be indicated by the author's name and the date, enclosed in brackets, thus [Ackermann, 1912, 1]. If reference is made to several papers published by one author in a single year, these should be numbered in sequence and the number quoted along with the year both in the text and in the collected references.

5. Chemical formulae should be written, as far as possible, in a single horizontal line.

6. Illustrations and curves accompanying the papers must be carefully drawn, about twice the size of the finished block, on smooth white Bristol boards in Indian ink. Any lettering on these drawings should be lightly inserted in pencil. Further information can be obtained from the Editors.

IX. AN INVESTIGATION OF THE METHODS EMPLOYED FOR COOKING VEGETABLES, WITH SPECIAL REFERENCE TO THE LOSSES INCURRED.

PART II. GREEN VEGETABLES.

By HELEN MASTERS and PHYLLIS GARBUTT.

From the Household and Social Science Department, King's College for Women.

(*Received January 15th, 1920.*)

These investigations were undertaken, as indicated in Part I [Masters, 1918], primarily with a view to obtaining some comparison of the different methods of cooking vegetables, with special references to the losses incurred and the time required for cooking. It was hoped that in this manner some method of cooking might be devised, which would be economical both as regards food material and as regards fuel, and that some further information concerning the changes which take place during the cooking process might be obtained.

Katherine Williams [1892] gives the results of a number of analyses of cooked and uncooked vegetables, but does not indicate how these results would be modified by different methods of cooking.

Other observers [Harden and Zilva, 1918; Delf, 1918] have shown that the antiscorbutic properties of vegetables may be destroyed by the action of dilute alkaline solutions or of heat. This is a physiological problem and the question whether or not it is advisable from a dietetic point of view that heat or alkaline solutions should be used in the preparation of vegetables hardly comes within the scope of this paper. It is hoped, however, that these investigations may prove of some assistance in arriving at a decision on such matters.

Numerous kinds of green vegetables are made use of for edible purposes, but the varieties available vary according to the season of the year and it was found impossible to work with the same variety throughout. To obviate this difficulty as far as possible, a number of experiments were made with each variety, and the nature of the vegetable used is stated in each case; but this variation in the nature of the material has hampered the work considerably and increased the difficulty of obtaining reliable results. The vegetables principally employed were cabbage, spring greens and Brussels sprouts.

In cooking green vegetables it is necessary not only to consider the losses on cooking and the time of cooking, but also the extent to which it is possible to preserve the original green colour of the vegetable, since no method of cooking can be considered satisfactory from a practical point of view unless the vegetables are a "good colour."

Some investigations were therefore first made of the colour changes which occur when green vegetables are cooked under various conditions.

Colour changes produced in green vegetables on cooking.

It appears to be fairly generally accepted that the preservation of the colour of green vegetables during the process of cooking can best be effected in the following ways:

(1) By cooking in a considerable volume of rapidly boiling water in an *open* vessel, *i.e.* in a vessel without a lid.

(2) By the addition of a small amount of alkali, usually sodium bicarbonate, to the cooking water.

Preliminary experiments indicated that there was considerable foundation for these ideas. Vegetables cooked in a covered vessel were undoubtedly inferior in colour to those cooked in an open vessel. The addition of ordinary salt to the cooking water appeared to improve the colour slightly, but the difference was not very marked. The cooking water was slightly acid to litmus.

The addition of small amounts of sodium bicarbonate to the cooking water had a marked effect on the colour of the vegetables. A bright green colour, more pronounced than that of the uncooked vegetables, was produced in most cases. It was also noticed that the alkaline cooking water was dark in colour, and that the veins and stems of the leaves of the vegetable, which are usually white, were coloured green, as though the colouring matter had been spread or developed by the action of the alkali.

Conversely, it was noted that if a little acid, *e.g.* acetic acid, were added to the cooking water, the vegetables rapidly changed in colour and became brown, but that the cooking water was only slightly coloured.

Chlorophyll, it is stated [Allen, 1909], is soluble in alkaline solutions and is rapidly decomposed after the death of the cell, a transformation to a brownish green chlorophyllan occurring unless alkali is present; the brown colour of badly cooked vegetables is attributed to the formation of this substance.

A preparation of chlorophyll was examined and was found to be insoluble in water, but readily soluble in water containing a little sodium bicarbonate; the solution thus obtained gave no colour change on treatment with dilute acids, and it was thought possible that the chlorophyll had been specially treated to render the colour stable.

On extracting some of the fresh green vegetable with alcohol, however, a solution which was deep green in colour was obtained, and this colour was

rapidly changed to brown on the addition of a few drops of acetic acid. The same effect was produced with other acids, but with sulphuretted hydrogen (see below) the reaction was slow, the colour changing gradually on standing for a few minutes at the room temperature.

Thus, in order to preserve the green colour of the vegetables, the presence of any acid reacting substance in the water should be avoided. This may, of course, be effected by rendering the water alkaline by the addition of sodium bicarbonate. The other effects observed on the addition of the bicarbonate, *i.e.* the dark colour of the cooking water and the stained appearance of the white portions of the leaf, may be attributed to the solubility of the chlorophyll in the alkaline solution. (For the effect of the addition of sodium bicarbonate on the time of cooking, etc., see pp. 82, 83.)

The action of acid on chlorophyll, taken in conjunction with the fact that the cooking water was found to be acid unless sodium bicarbonate was added, seemed to indicate that acid substances might be produced from the vegetables on cooking. Such acids, if volatile in steam, would, to a great extent, be removed if the vegetables were cooked in an open vessel of rapidly boiling water, but would be mostly retained if a covered vessel were used, and this might afford an explanation of the differences in colour observed with vegetables cooked in open and in covered pans.

Further information as regards the production of acid substances on cooking therefore appeared to be needed, and experiments were made to investigate the nature of the acids produced.

Acid substances produced on cooking green vegetables.

In order to determine whether volatile acids were produced on cooking, green vegetables were cooked in a large distilling flask attached to a condenser, and the distillate was collected in portions of about 100 cc.

It was found that the distillate was at first acid but on continuing the distillation it afterwards became slightly alkaline.

Examination of the distillate.

Since the distillate was only slightly acid, it appeared hardly feasible to identify the acids present without some further process of separation and concentration. It was, therefore, proposed to separate the acids in the form of their lead salts, by adding lead carbonate to a considerable volume of the distillate. On the addition of lead carbonate, however, a brown coloration was obtained which seemed to indicate the presence of sulphide.

This was confirmed by adding a few drops of a dilute solution of sodium nitroprusside to another portion of the distillate, which had previously been rendered *slightly* alkaline by the addition of ammonia. The characteristic violet colour produced by sulphides was obtained.

Determination of volatile sulphide in the cooking water.

Since it seemed probable that the amount of volatile sulphide produced might vary with the nature of the vegetable, and also with the method of cooking, it appeared of interest to try and devise some method for obtaining a measure of the amount of sulphide present in the cooking water, and a number of experiments were made with this object in view. The amount of sulphide produced is very small in comparison with the weight of vegetable cooked. The composition of the vegetable itself is by no means constant even when the same type of vegetable is used, and it is difficult to obtain any very consistent results.

After several methods had been tried and found unsuitable, a method was finally devised which gave results of sufficient accuracy to be of value for practical purposes, but before describing this method and the results obtained, a brief summary of the other methods and the difficulties met with may be given here for the benefit of other observers.

In the first experiments, 100 g. of cabbage were cooked in water in a flask attached to a condenser and the distillate was collected in portions of 100 cc. in Nessler cylinders. The amount of sulphide in each portion was determined colorimetrically.

The amount of sulphide in the first portion of the distillate was small, increased as distillation proceeded and then decreased again becoming fairly constant, but it was impossible to obtain any definite end-point, and after a time the distillate became cloudy.

It seemed probable that sulphide would continue to be evolved as long as the cooking was continued, and it was noted that sulphide was produced even when the distillate was no longer acid.

In further experiments, the vegetables were cooked for a definite time only under a reflux condenser and the cooking water distilled after straining off the solid matter. It was found that the sulphide could not be completely separated from the cooking water by distillation and that some of the sulphide underwent decomposition during this process.

There was also a considerable loss of sulphide from the condenser during the first part of the experiment in spite of a device arranged to minimise such loss. These observations were confirmed by some experiments made with dilute solutions of hydrogen sulphide of known concentration, which showed that a large proportion of the sulphide was decomposed when such solutions were heated and distilled under conditions similar to those employed in the experiments.

It appears hardly possible to obtain a reliable measure of the amount of sulphide produced, during cooking, but it was thought that if some method, which did not involve a distillation process, could be devised, it might be possible to obtain a measure of the amount of sulphide left in the cooking water at the end of the experiment, and that if all experiments were carried

out under the same conditions such information might be of value for comparative purposes, even though it did not represent the total amount of sulphide produced during cooking. For this purpose, a modification of the method described by Winkler [1917] was adopted, and was found to give more consistent results than the methods previously described.

Method used for the determination of the volatile sulphide in the cooking water of green vegetables.

The vegetable was cooked as before under a reflux condenser for one hour. Carbon dioxide was bubbled through a measured volume of the cooking water carrying with it the volatile sulphide, which was absorbed in a solution of hydrogen peroxide and thus oxidised to sulphuric acid, the amount of which was determined by titration with sodium hydroxide after all the carbon dioxide had been removed by boiling.

The determination was made as follows: 100 g. of the vegetable were cooked in 750 cc. water under a reflux condenser for one hour. The cooking water was made up to a litre and 250 cc. placed in a flask connected with a carbon dioxide Kipp and an absorption apparatus consisting of three conical flasks, each containing 150 cc. of a 0·15 % hydrogen peroxide solution. Carbon dioxide was bubbled through the cooking water, carrying with it the hydrogen sulphide, which was absorbed by the hydrogen peroxide and oxidised to sulphuric acid. The contents of the absorption flasks were washed into a beaker, and after the carbon dioxide had been expelled by boiling and the solution evaporated to about one-eighth of its original bulk, the sulphuric acid formed was determined by titration with $N/100$ sodium hydroxide solution. Correction was in each case made for the acidity of the hydrogen peroxide solution.

Several experiments were made and the average results obtained were as follows:

Table I.

Nature of vegetable	g. of hydrogen sulphide in cooking water from 100 g. of vegetable
Cabbage I	0·0017
„ II	0·0015
Green leaf of cabbage	0·0049
White „ „	0·0011
Brussels sprouts I	0·0094
„ „ II	0·0084

The results indicate that there is considerable variation in the amount of sulphide produced from the two types of vegetable used in the experiments, the average results for the Brussels sprouts being considerably higher than those obtained for cabbage.

Some further experiments were made in this connection, in which the green portions of the cabbage were separated from the white portions, and

cooked separately, the amount of sulphide left in the cooking water being determined in each case (see Table I above).

These results show that sulphide is liberated from both portions, but that less is liberated from the white leaf than from the green. Thus the relatively high results obtained with Brussels sprouts may be partly accounted for by the relatively small portion of white leaf found in this vegetable.

In addition to determining the sulphide present in the cooking water, determinations of the nitrogen and sulphur contents of the vegetables and portions of the vegetables were made.

Determination of sulphur in cabbage.

The sulphur was determined by fusing the dried vegetable with sodium peroxide, the sulphate thus formed being subsequently precipitated and determined as barium sulphate [see the method described in the Official and Provisional Methods of Analysis issued by the United States Department of Agriculture].

Determination of protein.

The nitrogen was determined by the Kjeldahl process, on about 3 g. of the dried vegetable, and the results were calculated as protein on 100 g. of uncooked vegetable and not on the dried vegetable.

Table II.

	Nature of vegetable	g. sulphur present in 100 g. uncooked vegetable	g. protein in 100 g. uncooked vegetable
I.	Cabbage (whole)	0·160	1·9
	„ green portion	0·162	2·2
	„ white „	—	1·6
II.	Cabbage, green portion	—	1·9
	„ white „	—	1·5
I.	Brussels sprouts	0·135	3·9
II.	„ „	—	3·5

These results, when compared with those of Table I, indicate that there is no definite correspondence between the sulphur content and the volatile sulphide in the cooking water.

As regards the nitrogen content, the results obtained appear to be in agreement with those arrived at by other observers.

Dugast [1882] found that generally speaking the protein in the leaf of the vegetable was greater than that in the stalk and that in the former case it varied from 11·9–21·2 % and in the latter from 7·3–13·3 % calculated on the *dried* vegetable.

Comparison with Table I shows that the yield of volatile sulphide is greatest where the protein content is high and *vice versa*. This suggests the possibility that the sulphide is produced by the decomposition of protein material, and it may be noted in this connection that Retteger [1906] showed that volatile

sulphide is liberated from heated milk and attributes this to the partial decomposition of the proteins.

The possibility of the sulphide being produced from alkyl sulphides[1] was also considered and attempts have been made to isolate such bodies by extracting some of the dried vegetable with ether in a Soxhlet extractor; so far no indication of their presence has been obtained, but further investigations in this connection are needed.

Other volatile acids produced on cooking.

In the experiments thus far described, the production and determination of volatile sulphide has been principally considered, but the possibility of the liberation of other volatile acids during the cooking process was also borne in mind and in some of the preliminary experiments the acidity of the distillate from the cooking water as well as the amount of sulphide present were determined. The results obtained for the acidity showed considerable variation even when samples of the same vegetable were used, the differences being still more marked when the experiments were made with different vegetables. It has been shown in the foregoing that such variations are almost inevitable in experiments of this character. In spite of this variation in the determined acidities, some evidence was obtained that the acidity of the distillate was greater than could be entirely accounted for by the volatile sulphide present.

Other observers have shown the presence of such acids as acetic and formic in vegetables. Borgmann [1883] found that a great number of plants, both in the presence and in the absence of chlorophyll, contained formic and acetic acids, and that in the case of chlorophyll-bearing plants the percentage of acids rises when assimilation is repressed.

Nelson and Beck [1918] showed that propionic and acetic acids could be isolated from fermenting cabbage (*sauerkraut*) and that in two cases formic acid had also been detected.

It, therefore, appeared by no means improbable that volatile organic acids might be liberated from green vegetables during the process of cooking. In order to verify this, it was first necessary to devise some method of removing the volatile sulphide in such a manner that other volatile acids would be left in solution.

Some experiments made with dilute solutions of formic and acetic acids containing hydrogen sulphide showed that, if the sulphide were expelled by bubbling carbon dioxide through the solution, in the manner previously described, the excess of carbon dioxide could be removed without loss of the acetic and formic acids, by heating the solution under a reflux condenser for about half-an-hour. If the time of heating exceeded one hour there was a slight loss of volatile acid, but heating for half-an-hour was found to be sufficient to expel the carbon dioxide. This method was next applied to the examination of the cooking water in which green vegetables had been cooked.

[1] Tibbles [1912] states that most of the Cruciferae contain allyl sulphides.

Examination of the cooking water for acids other than hydrogen sulphide.

The vegetable was cooked in a flask attached to a condenser for one hour. The distillate was diluted to a measured volume with distilled water and divided into two portions. The acidity of one portion was determined directly by titration with standard acid. The volatile sulphide was removed from the other portion and the acidity of the solution then determined by titration.

It is, perhaps, necessary to point out that such determinations do not, for the reasons already stated, give any exact measure of the volatile acids produced on cooking, but it was thought that they might furnish some evidence as to the presence or absence of volatile acids other than hydrogen sulphide.

The acid in the distillate from 100 g. of cabbage was found on the average to require 0·014 g. of sodium hydroxide for complete neutralisation, whilst after the removal of volatile sulphide the sodium hydroxide required for neutralisation averaged 0·005 g. Thus the solution is still slightly acid after the removal of the volatile sulphide, but the amount of acid present is extremely small and it is difficult to obtain consistent results.

Qualitative tests for formic and acetic acids were also carried out with this distillate, and some indication of the presence of these acids was obtained, but in dealing with such small quantities these tests can hardly be regarded as conclusive.

Total acidity of the cooking water.

In addition to determining the volatile acidity of the cooking water, some determinations of the total acidity were made. Owing to the dark colour obtained on the addition of alkali, phenolphthalein could not be used as indicator, but it was found that if turmeric paper were used as an outside indicator a moderately good end-point could be obtained. The acid in the cooking water from 100 g. of cabbage was found to require, on the average, 0·055 g. of sodium hydroxide for complete neutralisation. Thus the total acidity of the cooking water is considerably greater than the volatile acidity measured as described above. This may be partly accounted for by the loss of volatile sulphide on distillation and also the difficulty of separating the volatile acids completely by distillation, but it is also possible that non-volatile acid substances are present in the cooking water and further investigations are in progress.

The addition of sodium bicarbonate to the cooking water.

If sodium bicarbonate is added to the cooking water, any acids produced during the cooking process are neutralised and the cooking water at the end of the experiment is alkaline. It was found that if this alkaline cooking water were distilled, using a trap as in a Kjeldahl determination to prevent non-volatile alkali passing over, an alkaline distillate which contained appreciable quantities of sulphide was obtained.

It was observed that the vegetables cooked and disintegrated more rapidly in the presence of alkali. This is possibly because the cell walls are composed of a hydrated form of cellulose, which is more or less soluble in alkaline solutions [Cross and Bevan, 1903]. It also seemed possible that the amount of sulphide produced would be greater than when water only was used, but owing to the difficulty of determining such small quantities of sulphide in an alkaline solution, it is not possible at present to make any definite statement on this point.

As regards the alkalinity of the distillate from the cooking water when sodium bicarbonate is used, it should be noted that it was observed that the distillate from the cooking water became alkaline after a time, as the cooking proceeded, even when bicarbonate was not employed.

It was thought that this alkalinity might possibly be due to the evolution of ammonia or substituted ammonias formed as decomposition products during cooking, and that, if this were the case, the ammonia would probably be liberated more rapidly from a solution containing alkali.

Some preliminary experiments appear to give support to this theory, as some evidence has been obtained that the amount of alkali liberated is considerably greater when sodium bicarbonate is employed. These results, however, need further confirmation.

Summary and Conclusions.

The changes in colour observed on cooking green vegetables appear to be chiefly due to the action of acids liberated during cooking on the chlorophyll, the characteristic green colour of which is destroyed by the action of acids. During cooking, hydrogen sulphide and probably also volatile organic acids are liberated; these acids may to some extent be neutralised by other products of decomposition, but unless sodium bicarbonate is added, the cooking water is acid at the end of the experiment.

Green vegetables are usually cooked in an open vessel containing rapidly boiling water, and in this way there is a maximum loss of volatile acids, but if, on the other hand, they are cooked in a covered vessel then these volatile acids are retained in the vessel for a time at least. Although the volatile acidity is comparatively small, it is possible that the action of acids on the chlorophyll may be specially harmful during the earlier stages of cooking, and hence conditions which tend to prevent a free and rapid loss of volatile acids have a deleterious effect on the colour of the cooked vegetable, and this probably explains the poor colour of green vegetables cooked in a covered vessel.

It may also be noted that many cooks advocate the use of a large volume of water for cooking in order to preserve the colour; this would have the effect of diluting the acids produced.

If sodium bicarbonate is added, the cooking water does not become acid although volatile sulphide is produced. The neutralisation of the acids pro-

duced during cooking does not, however, entirely explain the action of the bicarbonate. The alkali has a solvent action on the chlorophyll, and also, probably because of its action on the plant cellulose, it causes a decrease in the time required for cooking. This latter effect also tends to improve the colour, since prolonged cooking renders the vegetables a bad colour.

In dealing with the action of bicarbonate, it is perhaps necessary to draw attention to a popular fallacy. It is frequently stated that the bicarbonate is added to soften the water and that this renders the vegetables a good colour. Careful observations, however, show that the colour of green vegetables cooked in really soft water, *i.e.* distilled water, compares unfavourably with that of those cooked in ordinary London tap water. This difference is, in all probability, due to the alkalinity of the tap water.

Losses incurred during cooking and Time required for cooking.

The question of colour having been dealt with, a comparison of the different methods of cooking as regards losses incurred and time of cooking was next considered.

The methods most frequently employed for cooking green vegetables appear to be the following:

I. Boiling in water, with or without the addition of sodium bicarbonate and salt.

II. Steaming.

III. Conservative method.

Vegetables were cooked by each of the above methods and the time of cooking, loss of solids, and loss of mineral matter compared.

As mentioned in Part I, it is difficult in carrying out experiments of this type to obtain consistent results, owing to variations in the nature of the material used for the experiments, the absence of any very definite end-point, and the difficulty of working under exactly similar conditions without adopting precautions which would not ordinarily be employed, and which, if introduced, might render the results less valuable from a practical point of view.

In the case of fresh green vegetables, the variation in composition is much more marked than with the dried vegetables dealt with in Part I; also, as already stated, the type of vegetable available varies according to the season of the year. Thus it is often impossible to repeat experiments, or confirm results obtained at an earlier period of the investigations. For these reasons, it was deemed advisable to carry out several experiments in each case and to take an average and also, in comparing the different methods of cooking, to make a control experiment with each sample of vegetable, by boiling a portion in water and comparing the results with those already obtained.

The methods adopted for cooking were as follows.

I. *Boiling in water.*

(*a*) 100 g. of the vegetable were washed with cold water in a strainer and were then immersed in 1000 cc. of boiling water, heated in an open pan and the boiling continued until the vegetables were quite soft as indicated by gently piercing with a fork. The time of cooking was noted. The vegetables were strained off, the cooking water evaporated to dryness on a water-bath and the residue heated in a steam-oven until a constant weight was obtained. This gave the weight of solid matter lost during the cooking process. The residue was then ignited over a flame until completely ashed, to obtain the weight of mineral matter.

(*b*) *The addition of sodium bicarbonate to the cooking water.*

The above experiments were repeated with the addition of 0·5 g. of sodium bicarbonate to the cooking water. The weights of residue and mineral matter were determined as before, a correction being made for the sodium bicarbonate added.

(*c*) *The addition of salt to the cooking water.*

The above experiments were repeated with the addition of 2·0 g. of salt to the cooking water. The weights of residue and mineral matter were determined as before, a correction being made for the sodium chloride added.

II. *Steaming.*

100 g. of the vegetable were washed with cold water in a strainer, and placed in a steamer, covered with a lid, over a pan of boiling water. The cooking was continued until the vegetables were soft, and the time of cooking noted.

The vegetables were allowed to drain for a few minutes over the pan and were then removed from the steamer. The steamer was washed down with a little distilled water, and the washings added to the cooking water. The solids and mineral matter in the cooking water were determined as described above.

III. *Conservative method.*

In the so-called "conservative" method of cooking vegetables, the vegetables are cooked in a very small quantity of water, so that the water is all absorbed by the vegetables during the cooking process. The vegetables are served without straining and there is practically no loss on cooking except volatile matter. 100 g. of the vegetable were covered with about 50–60 cc. of water and gently heated in a covered pan until quite soft. The time of cooking was noted.

The average results obtained for the different methods are given below (Table III). It should be noted, however, that the uncooked vegetable used for the experiments contains a large proportion of water, on the average about 89 %, the residues obtained therefore represent the loss on 100 g. of uncooked vegetable or on 11 g. of solid matter on the average, and the results

have been expressed as the loss both on 100 g. of vegetable and also on 100 g. of solids.

Table III.

Nature of vegetable	Method of cooking	Time of cooking	Loss of solids (*a*) on 100 g. of raw vegetable	Loss of solids (*b*) on 100 g. of solids	Loss of mineral matter (*a*) on 100 g. of raw vegetable	Loss of mineral matter (*b*) on 100 g. of solids
I. Cabbage	Boiling	30 mins.	4·45	40·45	—	—
,,	,, +0·5 g. sodium bicarbonate	20 ,,	3·37	30·64	—	—
,,	Boiling	30 ,,	3·19	26·94	0·689	5·82
,,	,, +0·5 g. sodium bicarbonate	20 ,,	2·26	19·10	0·438	3·70
,,	Boiling	35 ,,	3·81	39·64	0·437	4·55
,,	,, +0·5 g. sodium bicarbonate	22 ,,	3·41	35·48	0·421	4·38
II. Spring greens	Boiling	20 ,,	5·17	37·68	1·023	7·49
,, ,,	,, +2 g. salt	20 ,,	4·50	32·77	0·800	5·83
,, ,,	,,	25 ,,	3·39	33·50	0·803	7·95
,, ,,	,, +2 g. salt	30 ,,	3·49	34·55	0·892	8·83
III. Cabbage	,,	35 ,,	3·81	39·64	0·437	4·55
,,	,, +2 g. salt	30 ,,	3·55	36·94	—	—
IV. Cabbage	Boiling	45 ,,	3·80	41·99	0·428	4·75
,,	Steaming	1¼ hrs.	0·88	9·72	0·224	2·48
,,	Boiling	30 mins.	3·56	39·33	0·555	6·13
,,	Steaming	45 ,,	1·44	15·91	0·304	3·35
,,	Boiling	35 ,,	3·81	39·64	0·437	4·55
,,	Steaming	1 hr.	0·92	9·61	0·303	3·15

These results show that the addition of sodium bicarbonate to the cooking water decreases the time required for cooking (compare I, Table III) and that the loss in solid matter is slightly less. This latter effect is probably due to the shorter time required for cooking. (Compare the action of ammonium carbonate in steaming vegetables.)

The addition of salt does not appear to have any marked effect either on the losses or on the time of cooking.

As would be expected, the loss in solid matter when the vegetables are steamed is considerably less than when they are cooked in boiling water, though the time of cooking is somewhat greater. It should be noted, however, that in cooking with steam it is necessary to use a covered vessel in order to retain the steam, and this applies also to the conservative method of cooking. The vegetables have, therefore, in both cases a bad colour, this effect being probably enhanced by the relatively long time required for cooking, and both methods are, for this reason, open to objection from a practical point of view.

The addition of ammonium carbonate to the water used for steaming green vegetables.

The experiments previously described, in connection with the action of alkali on the colouring matter of green vegetables, seemed to indicate that if the steam could be rendered alkaline this difficulty might possibly be obviated. Some experiments were, therefore, made in which green vegetables were steamed over boiling water to which a little ammonium carbonate had been added.

Preliminary experiments showed that the colour of the vegetables was greatly improved in this way. The experiments also showed that, provided only small quantities of the carbonate were employed, the cooked vegetables did not taste or smell of ammonia. The addition of the alkali appeared, as in the case of the addition of sodium bicarbonate to the water used for boiling, to decrease the time required for cooking, and if too much were added the vegetables rapidly became slimy and disintegrated.

Several experiments were made in order to determine the most suitable proportions of ammonium carbonate to use.

These experiments showed that some vegetables disintegrated more rapidly than others, but that on the whole the best results were obtained when 0·5–1·0 g. of ammonium carbonate was added to 1000 cc. of water for steaming, and when 0·1–0·2 g. was added to 100 g. of vegetable for the conservative method.

The losses of solid and mineral matter when 100 g. of the vegetable were steamed by this method were determined and the average results obtained were as follows:

Table IV.

	Nature of vegetable	Method of cooking	Amount of ammonium carbonate added to 1000 cc. water	Time of cooking	Loss of solids		Loss of mineral matter	
					(*a*) on 100 g. of raw vegetable	(*b*) on 100 g. of solids	(*a*) on 100 g. of raw vegetable	(*b*) on 100 g. of solids
I.	Cabbage	Boiling	—	34 mins.	3·81	39·64	0·437	4·55
	,,	Steaming	—	1 hr.	0·92	9·61	0·303	3·15
	,,	,,	0·5 g.	18 mins.	0·48	5·01	0·157	1·63
II.	Cabbage	Steaming	—	1 hr.	0·90	8·17	0·261	2·37
	,,	,,	2·4 g.	15 mins.	0·47	4·26	0·148	1·34
III.	Cabbage	Steaming	—	30 mins. (not completely cooked)	0·52	4·72	—	—
	,,	,,	2·4 g.	30 mins. (slightly overcooked)	0·66	5·96	—	—

Conservative method.

	Nature of vegetable	Method of cooking	Amount of ammonium carbonate added to 60 cc. water	Time of cooking
I.	Cabbage	Boiling	—	30 mins.
	„	Conservative	—	1 hr.
	„	„	0·14 g.	30 mins.
II.	Cabbage	Conservative	—	1 hr.
	„	„	0·3 g.	12 mins.

The results show that the losses of solid and mineral matter are less than in the case of vegetables steamed in the ordinary manner without the addition of ammonium carbonate, and this in spite of the fact that the vegetables appear to disintegrate more rapidly when ammonium carbonate is used. The explanation of this result probably lies in the shorter time required for cooking in the presence of the alkali.

In order to illustrate this point some experiments were carried out in which portions of the same cabbage were cooked (*a*) over water, (*b*) over water containing excess of ammonium carbonate (2·4 g. of ammonium carbonate per litre). In the first experiment, the cabbage was cooked completely in both cases, the time of cooking noted and the loss of solid matter determined. In the second experiment, the cabbage was cooked for the same length of time in both cases and the loss of solid matter determined.

These results (see Table IV) show that if the time of cooking is increased, the losses increase, and if the vegetables are cooked for the same length of time only, the loss when ammonium carbonate is added is slightly greater than when water only is used.

Although the preliminary experiments indicated that the cooked vegetables had no appreciable taste or smell of ammonia, if only a small quantity of ammonium carbonate were used, it was thought advisable to carry out a few experiments in order to determine whether any considerable quantity of the ammonium salt were retained in the vegetable, as if this were the case its addition to the cooking water might be considered undesirable.

Determination of the proportion of the ammonium carbonate retained by the cooked vegetable.

The vegetables were cooked in a steamer of wire gauze suspended in the neck of a wide necked flask. The flask was connected with an upright condenser, the end of which dipped below the surface of a measured volume of standard acid contained in a beaker. 35 g. of the vegetable were used for each experiment. A weighed quantity of ammonium carbonate was added to the water in the flask and the water boiled for half-an-hour. The amount of ammonium carbonate left in the flask and the amount absorbed by the acid in the beaker were determined, and the difference between the sum of

these amounts and the amount of ammonium carbonate originally added was taken as a measure of the amount of ammonium carbonate absorbed by the vegetable (see Table V).

Table V.

Weight of cabbage	Volume of cooking water	Weight of ammonium carbonate added	Weight of ammonium carbonate left in flask	Weight of ammonium carbonate in beaker	Total ammonium carbonate in flask and beaker	Loss of ammonium carbonate
35	500	0·248	0·154	0·041	0·195	0·053
35	500	0·246	0·151	0·055	0·206	0·040
					Mean ...	0·046

The results indicate that the cooking water and distillate together contain most of the ammonium carbonate originally added, but a small proportion is lost and is presumably absorbed by the vegetable. It is possible that, by the time the vegetable has been cut up and dished ready for table, some of the ammonia at first absorbed will have escaped and that the proportion finally retained by the vegetable is less than that indicated by the experimental results.

Delf [1918] in dealing with the loss of antiscorbutic properties on cooking cabbage states that in general the loss is greater the longer the time of heating and that slow cooking at a low temperature is more deleterious than more rapid cooking at a higher temperature. It would be interesting if information could be obtained in order to show whether cooking for a short time in steam rendered slightly alkaline is less or more deleterious than cooking in steam without the addition of alkali for a longer period.

Summary.

A comparison of the different methods of cooking green vegetables shows that the addition of a small quantity of alkali to the cooking water, in addition to having a marked effect upon the colour, also decreases the time required for cooking, and slightly decreases the losses incurred; this latter effect being probably due to the decrease in the time of cooking.

The addition of salt to the cooking water appears to have no appreciable effect either on the time of cooking or on the losses incurred.

The losses of solid and mineral matter when the vegetables are steamed are less than when they are cooked in boiling water. Cooking by steaming and also by the conservative method in which there is practically no loss is, however, open to the objection that the vegetables are in both cases cooked in a covered vessel, and have, therefore, usually a bad colour; also the time required for cooking is greater than when the vegetables are boiled in water.

Both these difficulties may be obviated, and in the case of steamed vegetables the losses incurred reduced by the addition of a small amount of ammonium carbonate to the water from which the steam is generated. The

addition of excess of alkali, either of ammonium carbonate in steaming or of sodium bicarbonate in boiling vegetables, is to be avoided or the vegetables become slimy and tend to disintegrate. (See also Summary, p. 83.)

This work has been made possible owing to the receipt of a research grant by one of us (P. G.) from the Department of Scientific and Industrial Research, for which we desire to express our thanks.

REFERENCES.

Allen (1909). *Commercial Organic Analysis*, 4th Edition.
Borgmann (1883). *Bied. Centr.* **65.**
Cross and Bevan (1903). *Cellulose*, 2nd Edition.
Delf (1918). *Biochem. J.* **12**, 416.
Dugast (1882). *Annales Agronomiques*, 226.
Harden and Zilva (1918). *Lancet*, September 7.
Masters (1918). *Biochem. J.* **12**, 231.
Nelson and Beck (1918). *J. Amer. Chem. Soc.* **40**, 1001.
Retteger (1906). *Amer. J. Physiol.* **6**, 450.
Tibbles (1912). *Foods, their Origin, Composition and Manufacture.*
U.S. Dept. of Agriculture, Bureau of Chemistry, *Bulletin* No. 107.
Williams (1892). *J. Chem. Soc.* **61**, 226.
Winkler (1917). *Analyst*, **12**, 26.

X. ON THE THEORY OF GELS. II. THE CRYSTALLISATION OF GELATIN.

By SAMUEL CLEMENT BRADFORD.

(*Received January 27th, 1920.*)

In a previous paper [1918] it has been shown that the reversible gelation of the natural emulsoids is a crystallisation process resulting from the viscosity of these substances and from their very small diffusion constants which greatly retard the crystallisation of the cooling sols, allowing the accumulation of a large excess concentration. Thus the two factors K and P in von Weimarn's formula are both so large that, with a small value of L, a very great number of crystallisation centres are produced, and a gel is formed. Detailed examination of the theory showed that it was sufficient to explain the numerous properties of gels. It was also pointed out that complex organic substances and those which are highly aggregated in solution tend to crystallise in the form of spherites, built up of a great many crystalline needles arranged radially, the interstices being filled with liquid giving the appearance of a globular crystal. The inference was drawn that organic gels are usually composed of submicroscopic spherites bathed in the mother-liquor from which they have crystallised, though in certain cases the ultimate crystalline particles may be of other forms.

This conception of gelation is in complete agreement with recorded experimental results: the variation in intensity of the Tyndall cone observed by Arisz [1915]; the development of submicrons seen by Menz [1909] in gelating 2 % gelatin, which increased from 4 or 5, showing Brownian movement, in a square division of the field with a side of 9μ, to 80 or 100, at rest, in the same space; the actual observation by Hardy [1912] of microscopic spherites, *i.e.* 10μ, in reversible carbon tetrachloride gels of 5-dimethylaminoanilo-3 : 4-diphenyl*cyclo*pentene-1 : 2-dione; and the globulitic ultramicroscopic structure of natural organic hydrogels from which the water had been expressed, recorded by Bachmann [1911]. Moreover it was shown that gelatin can readily be precipitated by alcohol in the form of spherites.

The utility of von Weimarn's theory is not confined to the explanation of the colloid state. It indicates, further, the conditions necessary to obtain a given substance in any desired form. If his formula explains the usual occurrence of gelatin in the colloid condition, it should be possible, by suitable modification of the factors, to reduce the number of crystallisation centres sufficiently to obtain microscopic crystals of gelatin from water. The form in which such crystals would be expected to occur is that of spherites.

Since Bütschli [1896] found that the liquid expressed from 5 % gelatin gels contains from 0·16 to 0·46 % of gelatin, the solubility of this substance in water must be about this value. To obtain larger crystals of gelatin it should be necessary merely to reduce the concentration of the crystallising sols to about this degree. Hydrosols of Nelson's "Crystal" leaf gelatin were made of 0·5 and 0·3 %. These were quickly heated to 100° and filtered into small crystallising dishes which had been previously cleaned with chromic acid mixture. The dishes were covered with lids and the liquid allowed to evaporate spontaneously at room temperature. The sols were only very slightly opalescent at first, but, particularly in the case of the 0·5 % sols, the opalescence soon developed to an extraordinary degree, reaching a dense milky, and almost opaque, appearance by the third day. After a few days the cloudiness disappeared and a faint light buff-coloured precipitate was seen on the base of the vessels. The amount of this precipitate slowly increased.

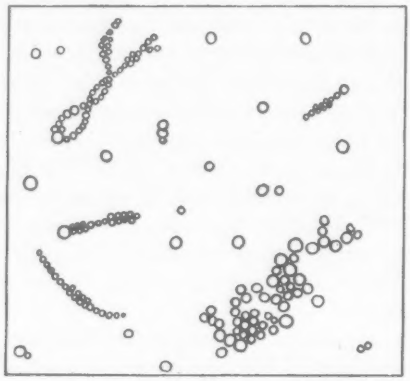


Fig. 1.
Gelatin spherites. ×800.

After about 30 days the supernatant liquid was poured off and the preparations examined under the microscope. In addition to numerous spherites there were, however, many bacteria present. The experiments were, therefore, repeated in the presence of 0·1 % mercuric chloride[1] with similar results, except that bacteria were entirely absent and the spherites perhaps more numerous.

The buff-coloured precipitate was examined most conveniently on the base of the dish with a $\frac{1}{15}''$ oil-immersion objective and 8 × eyepiece giving a magnification of about 800. That from the 0·3 % sols usually gave the best results. The diagram shows the appearance. Numerous single spherites were seen, mostly of apparently about 2·0 to 2·2 mm., or actually from 0·25μ to 0·28μ in diameter. In addition there were many small and some large aggregates of spherites. A number of typical ones are shown in Fig. 1,

[1] The molecular theory of solution [Bradford, 1919] gives reasons for supposing that dilute $HgCl_2$ would have little effect on the crystallisation of gelatin from water.

usually not more than two or three would be visible at once. The smaller spherites appear to prefer aggregation into filaments of which the individual elements were fairly distinct, but perhaps not quite as marked as in the diagram. The aggregates of larger spherites were usually in the form of colonies. Frequently the filaments appeared to originate from a large spherite, as shown, very much in the same way, on a smaller scale, as filaments often appear to have grown from spherites of silver dichromate and other inorganic substances. Except for the filaments and the more numerous detached spherites, the whole appearance is very similar to that of gelatin precipitated from a dilute sol by alcohol, or to the globulitic appearance observed ultramicroscopically by Bachmann, and to the spherites of mastic and gamboge obtained by Perrin.

Having regard to the appearance at first of the remarkable opalescence and its diminution as the precipitate appears, it cannot be doubted that gelatin crystallises from water exactly as indicated by von Weimarn's theory. The only possible complication in the cooling of the hot sols is that gelatin may melt in contact with water at a comparatively low temperature, so that the first separation may be into two liquid phases, one rich and the other poor in gelatin. Subsequent crystallisation from the more concentrated phase would result in still smaller submicrons.

In conclusion the writer wishes to express his indebtedness to Prof. J. B. Farmer for the kind loan of a high power microscope.

REFERENCES.

Arisz (1915). *Koll. Chem. Beihefte*, **7**, 1.
Bachmann (1911). *Zeitsch. anorg. Chem.* **73**, 150.
Bradford (1918). *Biochem. J.* **12**, 351.
—— (1919). *Phil. Mag.* **38**, 696.
Bütschli (1896). *Ueber die Bau quellbarer Körper*. Göttingen.
Hardy (1912). *Proc. Roy. Soc.* A. **87**, 29.
Menz (1909). *Zeitsch. physikal. Chem.* **66**, 137.

XI. A NOTE ON THE EFFECT OF PURGATION ON THE CREATININE CONTENT OF URINE.

By DAVID BURNS.

From the Department of Physiology, the University of Glasgow.

(*Received February 16th, 1920.*)

Anderson and Bosworth [1916] found that the administration of inositol to man, in doses of about 0·5 g. per kilogram of body weight, produced an increased excretion of creatinine, both immediately after the initial dose and during the period succeeding the administration of this substance. They offer no explanation of this increase. (It is regrettable that they employed a diet containing meat as it has been demonstrated that the amount of creatinine excreted has no definite ratio to the amount of flesh ingested [Burns and Orr, 1916].) From their results two points bearing on this subject may be noted:

(*a*) Inositol is only to a slight extent absorbed by man and has no appreciable effect (except creatinine increase) on metabolism.

(*b*) The ingestion of inositol was invariably followed by diarrhoea.

Burns and Orr [1914], in studying the effect of copious ingestion of water on the nitrogen metabolism of man, came to the conclusion that the ingestion of water in large amounts either had no appreciable effect on the creatinine excretion or caused (one experiment) a temporary increase. Most of their subjects of experiment had, on the first day of increased water ingestion, rather loose stools. In one instance, the administration of three litres of water at one time caused such severe diarrhoea that the experiment had to be abandoned. The results of this experiment are appended (Table I), from which it will be seen that the creatinine excretion rose from 1339 mg. to 1385 mg. per day.

Table I.

Effect of diarrhoea induced by an excessive intake of water.

Total N.	Creatinine	Creatine	Notes
gm.	mg.		
12·8	1339	Nil	
13·6	1339	,,	
13·1	1385	,,	3 litres of water extra

The following experiments were designed to show how far the purging effect of inositol might be answerable for the creatinine increase.

Two subjects, B. and W., were employed. They had been often on experimental diets and could, therefore, be relied on to adhere strictly to the routine laid down. The diets prescribed (Table II) were creatine-creatinine-free. Urine was collected in 24 hour periods, made up to a volume of 2000 cc.,

and analysed immediately. After a suitable pre-period the subjects took three teaspoonfuls of Rochelle salts (or in Exp. 5 sodium bicarbonate and in Exp. 6 acid sodium phosphate) in warm water at 11 a.m. and 2 p.m. on one day. The collection of urine was continued for a day or two thereafter.

Results. Detailed results are appended in tabular form (Tables III, IV and V).

Nitrogen. The amount of nitrogen excreted by the kidneys varied but slightly. On the day after the purge the amount was somewhat less than on the preceding days, due probably to a retention of nitrogen succeeding the loss of nitrogen by the bowel on the day before.

Creatinine. In the first four experiments the creatinine output was increased on the day of the purge. In Exps. 1 and 2 (Table III) this increase

Table II.

Diets used during experiments.

Expt. 1. Breakfast: Tea—two cups with milk and sugar
Bread—one slice brown and one white, butter
Fruit
Lunch: Four slices bread and cheese
250 cc. milk, 50 cc. water (hot)
Essence of coffee
Tea: As breakfast
Supper: Porridge and milk (250 cc.)

Expt. 2. Higher nitrogen intake
Same as for *Expt.* 1 with the following exceptions:
Breakfast: Cocoa instead of tea and one egg added
Dinner and Supper: 300 cc. milk instead of 250 (no added water)

Expt. 3. Higher calorie intake

Bread	650 g.
Potatoes	300
Rice	200
Fruit	100
Margarine	50
Jelly	50

Porridge and milk, two eggs, chocolate (measured)
Tea and cocoa (measured)

Water Expt. As *Expt.* 1, *i.e.*

Protein	110 g.
Carbohydrate	325
Fat	67

Table III.

Effect of Rochelle salts per os on urinary creatinine.

	Day	Reaction	Total N. (g.)	Creatinine (mg.)	Notes
Expt. 1.	Subject B.				
	1	Neutral	12·5	1680	
	2	Neutral	12·8	1675	
	3	Neutral	12·7	1680	
	4	Alkaline	12·7	1690	Rochelle salts in warm water
	5	Slightly alkaline	11·9	1810	
Expt. 2.	Subject B.				
	1	Neutral	15·1	1680	
	2	Alkaline	15·1	1700	Rochelle salts in warm water
	3	Alkaline	12·9	1780	
	4	Neutral	14·98	1690	
	5	Neutral	14·7	1700	
Expt. 3.	Subject W.				
	1	Acid	12·96	1152	
	2	Acid	10·83	1142	
	3	Alkaline	10·33	1362	Rochelle salts in warm water
	4	Acid	9·83	1100	

was followed by a still further increase. In Exp. 3 the creatinine excretion on the day following the administration of Rochelle salts was slightly subnormal. After NaH_2PO_4 a decreased creatinine elimination was observed (Exp. 5, Table V).

Table IV.

Effect of $NaHCO_3$ per os on urinary creatinine.

Expt. 4. Subject W.

Day	Reaction	Total N. (g.)	Creatinine (mg.)	Creatine	Notes
1	Neutral	10·4	1640	Nil	
2	Neutral	10·4	1640	,,	
3	Acid	10·5	1680	,,	
4	Alkaline	10·6	1640	,,	8 g. $NaHCO_3$
5	Alkaline	10·89	1634	,,	(no purging effect)
6	Acid	10·89	1640	,,	

Table V.

Effect of NaH_2PO_4 per os on urinary creatinine.

Expt. 5. Subject W.

Day	Reaction	Total N. (g.)	Creatinine (mg.)	Creatine	Notes
6	Acid	10·89	1640	Nil	
7	,,	12·18	1630	,,	16 g. NaH_2PO_4
8	,,	11·4	1550	,,	
9	,,	11·01	1620	,,	

To summarise; diarrhoea induced by:

1. excessive water ingestion,
2. irritation of intestinal wall by an inert substance (inositol—Anderson and Bosworth),
3. inrush of fluid into the intestine by exosmosis from the tissues (Rochelle salts),

is accompanied by an increase in the creatinine content of the urine.

This alteration in the creatinine output might at first sight seem to be associated with an alteration in the $H^{\cdot}$ concentration of the urine. Underhill [1916, 1] has reported a series of experiments on rabbits and dogs in which he demonstrates a causal connection between acidosis[1] and creatinuria, and indisputably proves that relief of the acidosis by the administration of alkali leads to a decrease or even to a cessation of the elimination of creatine. The apparent connection between creatine and creatinine is apt to cause one to take for granted that on a creatine-fixed diet the appearance of creatine in the urine is accompanied by a fall in the amount of creatinine excreted—a totally gratuitous assumption. Underhill [1916, 2] in a second paper proves that while the administration of alkali prevents the elimination of creatine in the earlier period of a starvation acidosis, it does not materially affect the creatinine output. In Exp. 4, W. took 8 g. of $NaHCO_3$ on one day. No diarrhoea resulted and there was no appreciable alteration in the creatinine output. Acid sodium phosphate in a dose of 16 g. reduced the creatinine content of the urine but did not cause the appearance of creatine.

[1] Acidosis is in this paper used in the clinical sense of the term.

Two reasons may be put forward as explanation of this increased creatinine excretion after diarrhoea:

(*a*) Increased tone of the visceral musculature.

The evidence of the connection of tone of striped muscle with the creatine content of muscle and with the creatinine content of urine seems conclusive [Pekelharing, 1911; Pekelharing and Harhink, 1910; Pekelharing and van Hoogenhuyze, 1916, 1, 2; Cathcart, Henderson and Paton, 1918; Riesser, 1916; Stenvers, 1917].

No evidence has however been brought forward to show that visceral muscle, under sustained contraction, leads to the elimination of increased amounts of creatinine. Further, the increased peristalsis of diarrhoea does not necessarily indicate increased tone of the visceral musculature. It has been proved that muscular work, apart from sustained contraction, does not cause an increased creatinine excretion.

(*b*) The sensation of well-being experienced after a purge leads one to the conclusion that the organism, having been freed from by-products and residues, is in better working order, and, as a whole, improved in tone. The experiment in which sodium biphosphate was given cannot in fairness be brought in evidence against this, as the acidosis produced would cause a decreased creatinine elimination [Underhill, 1916, 1].

Summary.

Purgation by Rochelle salts or copious water ingestion leads to a slight increase in the amount of creatinine eliminated.

Administration of an alkali which does not induce purgation causes no appreciable alteration in the creatinine output.

The exhibition of an acid purgative (NaH_2PO_4) leads to a slight decrease in the creatinine content of urine (acidosis phenomenon).

I have to thank Prof. Noël Paton for helpful guidance and kindly criticism.

The expenses were defrayed by a grant from the Carnegie Trust for which I express my thanks.

REFERENCES.

Anderson and Bosworth (1916). *J. Biol. Chem.* **25**, 398.
Burns and Orr (1914). *Brit. Med. J.* 505.
—— (1916). *Biochem. J.* **10**, 495.
Cathcart, Henderson and Paton (1918). *J. Physiol.* **52**, 70.
Pekelharing (1911). *Zeitsch. physiol. Chem.* **75**, 207.
—— and Harhink (1910). *Proc. Amster. Akad.* **14**, 310.
—— and van Hoogenhuyze (1916, 1). *Kon. Akad. v. Wetensch. Natuurk. Afd.* **24**, 1577.
—— (1916, 2). *Kon. Akad. v. Wetensch. Natuurk. Afd. Proc.* **18**, 1564.
Riesser (1916). *Arch. exp. Path. Pharm.* **80**, 183.
Stenvers (1917). *Ned. Tijdssch. v. Geneesk.* **61**, 85.
Underhill (1916, 1). *J. Biol. Chem.* **27**, 127.
—— (1916, 2). *J. Biol. Chem.* **27**, 141.

XII. THE PREPARATION OF SÖRENSEN'S PHOSPHATE SOLUTIONS WHEN THE PURE SALTS ARE NOT AVAILABLE.

By CHARLES JAMES MARTIN.

From the Department of Experimental Pathology, Lister Institute.

(*Received February 17th, 1920.*)

I have found the following method useful and satisfactory when away from the resources of a well-equipped laboratory during the war, and it may be useful to others at the present time.

Recrystallise NaH_2PO_4, H_2O of the B.P. Dry in air between filter paper. Make a supposed $M/7{\cdot}5$ solution of the recrystallised salt. Titrate 10 cc. of this against $N/7{\cdot}5$ NaOH, adding 0·05 cc. of 0·5 % solution of phenolphthalein as indicator. Add the soda until, at 18° C., the colour is of the same intensity as that of a 0·0025 % sol of $KMnO_4$.

If exactly 10 cc. of the $N/7{\cdot}5$ NaOH is required, a $M/15$ solution of Na_2HPO_4 results. I have usually found, however, that slightly less than 10 cc. is required owing to imperfect drying of the recrystallised salt. We will call a the cc. NaOH required.

To make a litre of each phosphate solution proceed as follows:

(1) For $M/15$ NaH_2PO_4 dissolve $\frac{138{\cdot}1}{15} \times \frac{10}{a}$ g. of the recrystallised salt and make up to 1 litre.

(2) For $M/15$ Na_2HPO_4 dissolve $\frac{138{\cdot}1}{15} \times \frac{10}{a}$ g. of the recrystallised salt in about 500 cc., add 66·66 cc. of N NaOH and make up to a litre.

The proportion in which these two solutions should be mixed to obtain any hydrogen ion concentration between $P_H = 6$ and $P_H = 8$ (within which range they are usable) is given by Sörensen [1909] and by Walpole [1911, 1914].

Moulds grow in phosphate solutions readily and are particularly troublesome in hot climates. Most antiseptics are not permissible. I have obviated this source of trouble by shaking the solutions with a little calomel for a few minutes and then filtering.

REFERENCES.

Sörensen (1909). *Compt. rend. Lab. Carlsberg*, **8**, 1.
Walpole (1911). *Biochem. J.* **5**. 207.
—— (1914). *Biochem. J.* **8** 628.

XIII. THE INFLUENCE OF REACTION ON COLOUR CHANGES IN TYROSINE SOLUTIONS.

By ELFRIDA CONSTANCE VICTORIA VENN.

From the Research Institute in Dairying, University College, Reading.

(*Received February 24th, 1920.*)

The work which is described in this paper is a continuation of that which was previously carried out on colour changes produced by two groups of bacteria on caseinogen and certain amino-acids [Cornish and Williams, 1917]. In that paper it was demonstrated that certain groups of bacteria which had been isolated from discoloured Stilton cheese were capable of producing changes of colour on certain media, consisting of simple suspensions or solutions in water of caseinogen and certain amino-acids, which are among its degradation products and many of which are known to be present in ripe Stilton cheese. In the course of that work it had been frequently found that the constitution and reaction of the media upon which the organisms were grown appeared to exercise a very considerable influence upon the degree of discoloration, which tended to vary with the nature of the amino-acids present and to diminish with increased acidity.

Further tests were therefore carried out to study the influence of these two factors. Water solutions or suspensions which were either neutral or slightly acid were made of caseinogen, tyrosine, tryptophan, histidine, alanine and cystine and, in addition, agar media containing these substances were prepared and standardised to a reaction of -1 and -3 according to Eyre's method. The growths upon these media produced some striking colours which are recorded in Cornish and Williams' paper.

The present paper is concerned with further investigations designed to discover the effect of varying degrees of acidity and alkalinity upon the colours produced by one strain upon a single amino-acid. For this purpose a member of the Gram-negative Alk. group [Cornish and Williams, 1917] $C_{22}\,4^{B}$ was chosen, and its colour-producing effects upon tyrosine were studied. This strain $C_{22}\,4^{B}$ had been found capable of producing colour in histidine water, alanine water, caseinogen water, tyrosine water, tryptophan water and upon agar media containing respectively these amino-acids. Tyrosine was selected as the amino-acid partly because it is present in very considerable quantities in ripe cheese and is prepared with relative ease, and partly because it is a medium upon which colour changes are easily produced.

As a preliminary experiment a solution of 0·1 % tyrosine was made, and from this solutions of varying reaction from approximately + 15 to − 35 (Eyre's standard) were prepared by the addition of the requisite quantities of sodium hydroxide or hydrochloric acid. After inoculation, colours were produced in those media, of which the reaction lay between + 5 and − 35. In the latter case the colour was black, and in the former there was only slight darkening of the solution. The tubes which contained solutions of

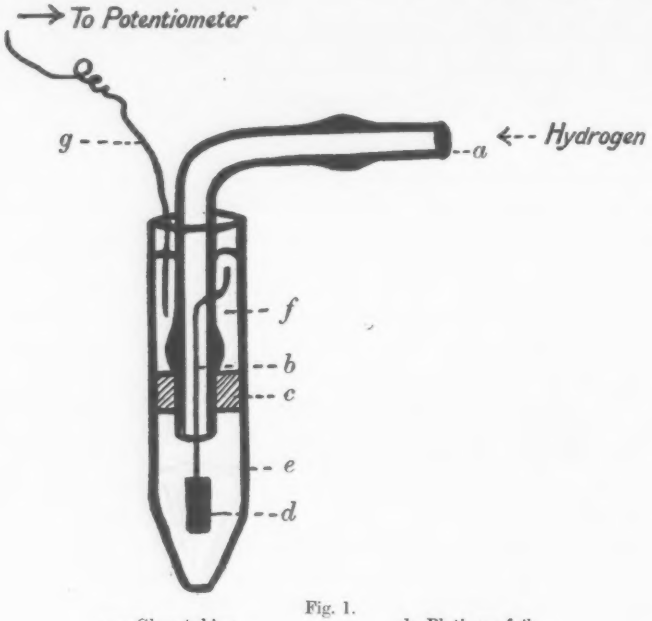


Fig. 1.

a = Glass tubing. *d* = Platinum foil.
b = Platinum wire. *e* = Glass cover.
c = Rubber band. *f* = Mercury cup.
g = Wire leading to potentiometer.

greater acidity than + 5 showed no change in colour and the controls remained unchanged. In view of these observations the series was then extended and solutions were made up with reactions which varied between − 35 and − 85. At the end of 27 days no colour change was found in those solutions with a greater alkalinity than − 45. These results seemed to indicate that there was a certain range of reaction within which this strain of bacilli was capable of acting upon tyrosine with production of colour, but beyond which colour changes were not produced.

In order to obtain a more accurate measure of the range within which colour changes may be produced, it was decided to carry out electromotive force determinations on a further series of inoculated and control tyrosine solutions.

The apparatus used in these investigations was a Tinsleys's universal potentiometer, a very sensitive d'Arsonval galvanometer, and as a calomel half electrode the saturated cell described by Barendrecht [1915].

For some time an endeavour was made to use the small hydrogen electrode also described by Barendrecht, but it was found very difficult to obtain consistent results with this type of electrode, owing no doubt to the nature of the solutions under investigation and the difficulty of cleaning the electrode. The simple hydrogen electrode described below was therefore devised, and for this particular piece of work has been found to give consistently satisfactory results.

The hydrogen electrode is shown slightly larger than actual size in the accompanying diagram (Fig. 1). (*a*) is a piece of glass tubing bent at right angles and slightly thickened as shown. (*b*) is a length of platinum wire, one end being fused through the glass and the other one carrying a platinum plate (*d*). The outer glass covering (*e*) is held in position by the rubber band (*c*) and the space above is filled with mercury forming the cup (*f*) by which contact is made to the potentiometer by wire (*g*) by simply inserting the wire into the mercury.

The advantages of this simple electrode are two. There is no dead space or side tube, and it is very easily and quickly cleaned and platinised. The disadvantage is the use of rubber tubing, but if this ring is placed in the position shown, the liquid under investigation need never come into contact with the rubber. The electrode is used in the same way as that of Barendrecht [1915] and shares its advantage of being small and therefore needing less time for saturation than a larger one. When a reading has been completed, the mercury is tipped out, the outer tube and rubber ring are taken off, and the electrode is cleaned and replatinised. This is done between each determination. It is found that there is no danger of leakage if the sizes of the glass and rubber tubings are carefully adjusted.

The results obtained by this method are given in the following table from which it appears that there is a definite range of reaction over which the organism studied is capable of producing darkening in tyrosine solutions, and beyond which such discoloration fails to appear.

In all cases except Nos. 30 and 31, the P_H was determined within a month of the date of inoculation of the solution, and a control uninoculated solution was also tested in 31 cases. Solutions Nos. 30 and 31 were kept for 14 months after inoculation before the P_H was determined, but at the end of that time no colour had developed and the P_H values were those recorded.

No explanation is offered of the fact that the inoculated portion of solution No. 29 with a P_H of 1·95 shows a slight colour. Apart from this single instance there appears to be a gradual increase of colour from P_H 3·23 passing through a maximum and gradually disappearing as the P_H approaches a value of about 9·7. The gradations in colour are not a perfect sequence, but if the whole series be considered it is found that, except in the case of No. 29, no colour

was produced at a P_H below 2·28; at 3·23 a lightish brown colour appeared which became a deep brown or reddish brown from 5·83 to 9·47. Above this point the colours are for the most part of a yellowish type until the limit is reached at 9·71.

Table of colour reactions produced in 0·1 % tyrosine solutions of varying P_H.

No.	Solution	P_H	Colour	No.	Solution	P_H	Colour
1	Inoculated	9·67	No colour	17	Inoculated	8·68	D.B.
	Control	9·71			Control	8·74	
2	Inoculated	9·63	V.Sl.Y. with	18	Inoculated	8·43	V.D.B.
	Control	9·59	V.Sl.P.		Control	8·57	
3	Inoculated	9·61	V.Sl.Y.	19	Inoculated	8·32	D.B.
	Control	9·63			Control	8·38	
4	Inoculated	9·61	V.Sl.Y.	20	Inoculated	8·20	D.P.B.
	Control	9·63			Control	8·60	
5	Inoculated	9·61	Cinnamon	21	Inoculated	7·51	D.B
	Control	9·63			Control	7·63	
6	Inoculated	9·55	Sl.P.Y.	22	Inoculated	6·69	D.B.
	Control	9·60			Control	7·15	
7	Inoculated	9·55	D.Y. with	23	Inoculated	5·83	V.D.B. with
	Control	9·58	P. tinge				Sl.P.
8	Inoculated	9·54	V.V.V.Sl.P.	24	Inoculated	3·23	L.B.
	Control	9·56			Control	(?)2·30	
9	Inoculated	9·54	V.Sl.Y.	25	Inoculated	2·59	No colour
	Control	9·53			Control	2·35	
10	Inoculated	9·50	P.B.	26	Inoculated	2·28	No colour
	Control	9·45			Control	2·47	
11	Inoculated	9·40	R.B.	27	Inoculated	2·26	No colour
	Control	9·40			Control	2·05	
12	Inoculated	9·39	V.V.Sl.	28	Inoculated	2·09	No colour
	Control	9·45			Control	2·05	
13	Inoculated	9·14	V.D.B.	29	Inoculated	1·95	L.P.B.
	Control	8·87			Control	1·93	
14	Inoculated	8·91	D.R.B.	30	Inoculated	1·72	No colour
	Control	9·20		31	Inoculated	1·71	No colour
15	Inoculated	8·87	R.B.	32	Inoculated	1·46	No colour
	Control	8·68			Control	1·63	
16	Inoculated	8·75	P.V.Sl.B.	33	Inoculated	1·32	No colour
	Control	8·43			Control	1·30	

Abbreviations: B = Brown; D = Dark; L = Light; P = Pink; R = Red; Sl = Slight; V = Very; Y = Yellow.

REFERENCES.

Barendrecht (1915). *Biochem. J.* **9**, 66.
Cornish and Williams (1917). *Biochem. J.* **11**, 180.

XIV. BARGER'S MICROSCOPICAL METHOD OF DETERMINING MOLECULAR WEIGHTS.

PART I. THE PRINCIPLE OF THE METHOD WITH REFERENCE TO THE MOLECULAR AND IONIC ATTRACTION OF SOLUTE FOR SOLVENT.

By KUMAO YAMAKAMI.

From the Department of Experimental Pathology, The Lister Institute, London.

(*Received February 24th, 1920.*)

A very reliable and convenient method has been described by Barger [1904] for determining molecular weights, which is explained by the author to be based upon the vapour pressure.

Its usefulness for studying the degree of association of acids, phenols, etc., when dissolved in mixtures of an associative and a non-associative liquid, has been proved by his interesting work which was published in the following year.

The technique of this method is quite simple. Series of drops, taken from two solutions, the one the standard, the other a given solution, of which the molecular weight of the solute is to be determined, are alternately introduced into a capillary tube, and the change in the length of the drops is studied by means of a micrometer. If one series of drops is observed to have increased while those alternating with them have decreased, it is to be assumed that the solution from which the former series of drops was taken has a higher molecular concentration than the latter.

The given solution is thus compared with a series of standard solutions of known molecular concentrations and in this way two limits are reached for the unknown molecular concentration.

The mechanism of this method is explained by Barger in the following way: "Each drop is placed between two others of a different solution and can evaporate on either side into a small air chamber. This closed air chamber is soon saturated with vapour, which can condense freely on the drops. If the vapour pressures of the two solutions are equal, the evaporation will equal the condensation, and there will be no change in the drops. If, on the other hand, the vapour pressures are unequal, there will be a gradient of the vapour pressure in the air spaces; some drops will therefore be in contact with an atmosphere, the vapour pressure of which is greater than their own.

Condensation will take place on these drops and they will increase. The others, alternating with them, have a vapour pressure greater than that of the adjoining air spaces; these drops will evaporate and thus decrease. Hence there is distillation from drops of the one series to those of the other series, although all are at the same temperature."

In the course of an investigation of caseinogen solutions by this method, I have observed some facts which made me think it impossible to ascribe the mechanism of this method solely to vapour pressure.

I was determining the molecular and ionic concentration of alkali caseinogenate solutions, employing urea solutions as the standard. But it was feared that urea might combine with the alkali contained in the caseinogenate solutions. As Barger states in his paper, substances which may combine with the solute of the solutions employed are not to be used as standards because a certain amount of mixing of the two solutions is inevitable. All the drops of both series are successively taken in from one end of the capillary tube, so that the wall of the tube is wetted by each drop alternately. Therefore, not only the drops of one series are contaminated with the other solution when they are taken in, but also it cannot be denied that the drops remain in contact with each other by means of a thin layer of a mixture of the two solutions. Hence it becomes necessary that the standard substance should not combine chemically with the substance of unknown molecular weight.

Under these circumstances, I tried to modify the method in such a way that this limitation in the choice of the standard substance might be removed.

At first sight this appeared to be very easy because I thought a satisfactory result would always be obtained if the two solutions were brought into a closed air space avoiding contact with each other.

But this was not the case. The volume change of solutions thus confined in an air-tight chamber was found to proceed extraordinarily slowly, if they were not allowed to touch each other, even when the two solutions were very close together. Such fairly regular and rapid change in volume of the solutions, as is seen in Barger's method, was very difficult to obtain.

The protocol of the experiments described in the following section illustrates this fact.

Experiment I. In the capillary tubes which are generally employed in Barger's method two small drops of solutions of different molecular concentrations were introduced from either end of the tube, so that the wall of the tube between the drops was left partly unwetted by the solutions. The lengths of the tubes were all 8 cm. The first drop was introduced in the usual way and allowed to slide down to the middle of the tube and then the other drop was introduced from the other end and allowed to slip down for a distance of 2 cm., the first drop, accordingly, going back to a point 2 cm. away from the other end. Each drop therefore stood 2 cm. away from its respective end and 4 cm. away from the other; 2 cm. of the wall of the tube between the drops remained dry, while the other 2 cm. were wetted by the first drop.

The ends of the tubes were then carefully sealed by heating. The tubes were then placed under water in Petri dishes, and the change of the drops at room temperature, 12°–16·5°, was studied every 24 hours, after the initial length of the drops had been measured immediately after the preparation of the tubes.

The results are given in Table I, in tenths of scale divisions, as explained below after Table III.

Table I.

No. of tube	Diameter of tube	Change in length of drop No. 1 (0·5 *M* urea solution)							
		Initial length	1st 24 h.	2nd 24 h.	3rd 24 h.	4th 24 h.	5th 24 h.	6th 24 h.	7th 24 h.
1	480	520	−10	−10	− 5	− 3	—	—	—
2	443	530	−12	−12	− 3	− 2	—	—	—
3	450	420	− 6	− 6	−10	− 8	—	—	—
4	400	413	− 8	− 7	− 5	− 5	—	—	—
5	510	212	−13	− 5	− 4	− 2	0	0	+ 2
6	467	242	− 8	− 3	− 3	+ 2	+ 5	0	+ 3
7	492	356	−11	−13	− 0	− 0	− 2	+ 1	− 1

No. of tube	Diameter of tube	Change in length of drop No. 2 (0·1 *M* urea solution)							
		Initial length	1st 24 h.	2nd 24 h.	3rd 24 h.	4th 24 h.	5th 24 h.	6th 24 h.	7th 24 h.
1	480	462	−46	−42	−40	−21	—	—	—
2	443	236	−41	−42	−15	−40	—	—	—
3	450	436	−48	−30	−35	−15	—	—	—
4	400	630	−42	−30	−18	−16	—	—	—
5	510	322	−47	−41	−24	−19	−15	− 8	−17
6	467	760	−30	−42	−25	−17	− 6	−15	− 9
7	492	449	−43	−27	− 5	−16	−18	− 7	− 5

It will be easily realized how quickly and regularly the change of drops takes place in Barger's method, if the above protocol is compared with the change of drops observed under normal conditions, as is shown in Table II.

Experiment II. 0·5 *M* solution of urea was compared with 0·1 *M* solution. In Table II the drops indicated by odd numbers are those of 0·5 *M* solution, and the even numbers denote the drops of 0·1 *M* solution. The lengths of the air spaces between the drops were also measured in order to make the relation of each drop clear (they are placed in brackets).

Table II.

No. of tube		Drops										Diameter of tube
		1		2		3		4		5		
1	(860)	585 +13	(925)	550 −12	(1150)	528 +8	(840)	600 −15	(870)	601 +9	(1200)	460
2	(950)	683 +14	(1080)	540 −15	(847)	522 +8	(705)	680 −14	(824)	521 +14	(915)	445
3	(1045)	487 +10	(1210)	479 −17	(920)	545 +17	(965)	569 −12	(1030)	500 +11	(995)	515

The initial measurement was made immediately after the preparation of the tubes, and the second determination was made 2·5 hours afterwards at room temperature, 15°.

As shown in Table II, the change in the lengths of the drops is quite decisive, the drops of the stronger solution increasing regularly and the drops of the weaker solution decreasing, only 2·5 hours after the preparation of the tubes, whereas such a change does not occur even after 100 hours at the same temperature and with the same solutions if the drops are 4 cm. apart and 2 cm. of the wall between the drops is left dry, as described in Exp. I, Table I. The difference between the drop-changes in these two cases seems too great to be attributed solely to the greater distance between the drops in Exp. I. That vapour pressure plays only a subsidiary part results also from experiments which were made with solutions of which an accurately known volume (about 0·4 cc.) was delivered on to weighed dry filter papers (3 × 12 cm.) placed parallel and face to face 6–7 mm. apart in stoppered glass cylinders at 37°. After 24 hours the cylinder was weighed before and after the removal of each paper separately, thus giving the weight of the papers by difference. Both papers invariably lost weight (0·06–0·15 g.) and no sort of regularity could be discerned unless the papers were allowed to touch after the solutions had been uniformly distributed in them. In that case it was possible to distinguish between 0·1 and 0·166, and between 0·1 and 0·05 molar concentrations, by the differences in the loss of weight.

These experiments with tubes and filter papers compelled me to doubt the importance of the vapour pressure in causing the change of drop-length in Barger's method.

The next experiment seems to prove definitely the existence of a factor other than the vapour pressure, the action of which appears to depend on the fact that the two solutions are in contact.

Experiment III. 0·5 *M* and 0·25 *M* aqueous solutions of urea, boric acid and cane sugar were prepared and the drop-change according to Barger's method was investigated in the two series.

Now, if the change of volume of the drops is produced solely by the difference in vapour pressures of neighbouring drops, this change must stop when the concentrations have all become equal, owing to the dilution of the drops of the stronger solution, and the concentration of the drops of the weaker solution by evaporation. The decrease in volume of drops taken from 0·25 *M* solutions must cease before their original length is halved, because their molecular concentration must be 0·5 at this point, if the decrease has taken place only by evaporation of the solvent.

If the drops of 0·25 *M* solution had ever been shown to have become just half of their initial volume, it would mean that distillation had proceeded from the stronger solution to the weaker, for the concentration of the 0·25 *M* drops would then be 0·5 *M*, while that of the drops of 0·5 *M* solution would be correspondingly weaker than 0·5 *M*.

This remarkable result has, indeed, been observed. The decrease in length of drops taken from 0·25 *M* solution actually proceeded until they were less than one-third of their original length, as shown in Table III (*a*), (*b*), (*c*).

It is impossible to explain this experimental observation without assuming that the drop-change occurred because of a transference, not only of vapour, but also of liquid from the weaker to the stronger solution, that is, the stronger solution attracted the solvent of the weaker solution through the film furnished by the liquid membrane which extended between the two drops. As the liquid membrane, differing from the semipermeable membrane which is generally employed in osmotic pressure experiments, is perfectly permeable for both solute and solvent, so the dissolved solute molecules have accompanied the solvent molecules to the stronger solution and consequently the 0·25 *M* drops were not correspondingly concentrated when diminution of their volume occurred.

Similarly, the dilution of the stronger solutions did not go parallel with the increase of their volume, which would be expected if the increase of the drops were caused by condensation of vapour only.

This seems to be the most probable explanation for the further increase of the volume beyond the point at which it would stop if the change depended solely on vapour pressure.

Table III (*a*)

30 hours at 35° C.

	1 (0·25*M*)		2 (0·5*M*)		3 (0·25*M*)		4 (0·5*M*)		5 (0·25*M*)		Diameter of tube
Urea	(850)	270 / 135	(750)	532 / 640	(1070)	264 / 140	(960)	600 / 740	(910)	254 / 110 (750)	455
„	(850)	304 / 140	(845)	684 / 890	(1020)	270 / 125	(860)	642 / 765	(1200)	230 / 101 (710)	515
Boric acid	(1000)	145 / 35	(850)	635 / 725	(990)	172 / 55	(888)	650 / 780	(940)	210 / 70 (775)	465

(*b*)

30 hours at 35° C.

	1 (urea)		2 (sugar)		3 (urea)		4 (sugar)		5 (urea)		Diameter of tube
Sugar, 0·5*M* Urea, 0·25*M*	(890)	193 / 35	(680)	521 / 670	(810)	274 / 95	(800)	630 / 800	(850)	290 / 110 (1000)	480
„ „	(890)	430 / 280	(1110)	360 / 510	(900)	395 / 240	(1030)	380 / 520	(1150)	460 / 310 (1300)	525
„ „	(1150)	304 / 151	(1000)	740 / 890	(860)	325 / 170	(1090)	695 / 850	(980)	220 / 55 (1060)	510

(*c*)

5 days at 13–16° C.

	1 (0·25*M*)		2 (0·5*M*)		3 (0·25*M*)		4 (0·5*M*)		5 (0·25*M*)		Diameter of tube
Urea	(895)	383 / 173	(945)	781 / 1004	(1005)	423 / 210	(1100)	867 / 1060	(975)	447 / 235 (1000)	445
„	(1030)	445 / 218	(965)	817 / 1030	(749)	390 / 177	(1010)	720 / 930	(940)	464 / 261 (920)	430
Boric acid	(910)	350 / 169	(744)	606 / 806	(780)	266 / 94	(772)	640 / 865	(916)	340 / 126 (850)	525

The measurement of the drop-length was made with the ocular micrometer 0·1 mm. No. 2 and Leitz objective No. 2.

One scale division of this micrometer corresponded to $\frac{1}{37}$ mm. when tested by means of a Carl Zeiss objective micrometer, 0·01 mm. The readings were made and are recorded throughout this paper in tenths of scale divisions. As the length of the intervals between the drops varied from 750 to 1400 tenths of a scale division, so the actual lengths of the intervals in the experiments were 2–4 mm., that is, a little greater than those indicated by Barger.

The fact that the 0·25 *M* drops decrease until they are less than one-third of their original length shows how small is the contribution of vapour pressure to the change of drops compared with the other factor.

In the following experiments the velocity of evaporation, which takes place from the surfaces of drops confined in glass tubes, was investigated in the case of aqueous solutions of various concentrations, by measuring the decrease in length after standing for some time at constant temperature.

The object of these experiments was to gain information as to the relative parts played by the vapour pressure and the second factor in changing the length of the drops in Barger's method.

Experiment IV. The leading idea of this experiment was that, if the vapour pressure is solely responsible for the change of drop-volume, then the difference between the velocity of evaporation of the weaker solution and that of the stronger solution must be equal to the magnitude of the volume-change in drops according to Barger's method, but if, on the other hand, the difference of these two velocities is not equal to the magnitude of the change, then the ratio of these two must represent the relative contribution of the vapour pressure and the other factor in bringing about the change.

The velocity of evaporation from the surface of the drops was found by measuring the decrease in their length when singly confined in capillary tubes.

The results thus obtained are presented in Table IV (*a*), (*b*), (*c*), (*d*).

Explanation of Table IV (*a*), (*b*). One small drop of distilled water was introduced into a capillary tube, both ends were carefully sealed and it was mounted on a glass slide, put in a Petri dish, and the decrease in length of the drop was observed at a constant temperature of 35°.

It was found that the drop thus confined in the capillary tube decreases in length fairly regularly with lapse of time. The length of the water drops decreased 80–96 tenths of a scale division during the first 24 hours. This appeared to be approximately the maximal amount which can evaporate from the surface of drops at this temperature and during this period, since increasing the length of the capillary tube did not result in a more rapid decrease of the drop-length. In an 8 cm. capillary tube, the amount of evaporation was not appreciably affected by the presence of another water drop near the other end of the tube.

This fact shows that the humidity of the air produced by evaporation during this period is not sufficiently great to influence appreciably the velocity

of evaporation. This conclusion is supported furthermore by the fact that the decrease in volume proceeds with almost the same speed during the next 24 hours.

In this experiment the approximate diameter of the capillary tube was measured, because it was feared that the surface dimension of the drops might not be exactly directly proportional to the diameter; in other words, the evaporating surface of a drop in a narrow tube might be relatively greater than that of a similar drop in a wider tube. But this apprehension proved to be unfounded. The amount of decrease was not influenced to any great extent by the difference of diameter within the variation of diameter of the tubes which we employed.

Table IV (*a*)

Single water drops in capillary tubes of varying length at 35° C.

No. of tube	Length of tube	Diameter of tube	Initial length of drops	Change in the 1st 2 hours	Change in the 1st 24 hours	Change in the 2nd 24 hours
1	6 cm.	450	726	− 4	−91	−85
2	8	460	962	− 8	−87	−90
3	10	455	453	− 6	−83	−88
4	12	400	775	− 5	−92	−94
5	14	430	680	−15	−98	−93

(*b*)

Two water drops in one tube (8 cm. long) at 35° C.

No. of tube	Diameter of tube	Drop No. I				Drop No. II			
		Initial length	1st 24 hours	2nd 24 hours	3rd 24 hours	Initial length	1st 24 hours	2nd 24 hours	3rd 24 hours
1	480	370	−86	−86	−79	466	−82	−80	−74
2	420	642	−85	−91	−69	440	−89	−81	−82
3	475	469	−86	−82	−67	303	−78	−76	−60
4	510	332	−84	−77	−74	496	−91	−85	−60
5	455	518	−87	−80	−71	421	−83	−83	−75

Explanation of Table IV (*c*), (*d*). When a substance was dissolved in the water, the velocity of evaporation expressed in terms of the diminution in length of the drops decreased with the concentration of the solution, as shown in Table IV (*c*), (*d*).

The amount of evaporation from drops of 0·5 *M* urea solution at a constant temperature of 35° in the first 24 hours varied from 28–39 tenths of a scale division, the mean decrease in length being 32.

Table IV (*c*)

Single drop and two drops of urea solution (0·5*M*) in one tube at 35° C.

No. of tube	Diameter of tube	Drop No. I				Drop No. II			
		Initial length	1st 24 hours	2nd 24 hours	3rd 24 hours	Initial length	1st 24 hours	2nd 24 hours	3rd 24 hours
1	480	469	−32	−12	− 8	—	—	—	—
2	410	332	−38	−23	− 3	—	—	—	—
3	505	308	−39	−21	−18	—	—	—	—
4	445	242	−31	−21	− 4	533	−39	−28	− 4
5	492	440	−34	−16	+ 2	442	−28	−18	−14
6	465	428	−30	−22	−11	415	−29	−24	− 8
7	460	302	−29	−14	− 6	320	−34	−16	− 2

The amount of evaporation from 0·25 *M* drops was found to be 53 tenths of a scale division as is shown in the Table IV (*d*).

Table IV (*d*)

Two drops of urea solution (0·25*M*) in one tube at 35° C.

No. of tube	Diameter of tube	Drop No. I				Drop No. II			
		Initial length	1st 24 hours	2nd 24 hours	3rd 24 hours	Initial length	1st 24 hours	2nd 24 hours	3rd 24 hours
1	420	244	−55	−36	−23	267	−44	−50	−32
2	440	501	−56	−35	−21	486	−52	−37	−20
3	510	320	−55	−37	−19	352	−57	−37	−11
4	435	382	−51	−42	−21	340	−54	−34	−26

Now if the change (increase and decrease) of volume of two drops in Barger's method is solely due to a difference of their vapour pressures, such volume-change should be equal to the difference between their decreases, when they are investigated separately as in Table IV (*a*)–(*d*), at the same temperature and for the same length of time.

The magnitude of change would, therefore, be expected to be about 87·4 − 32·0 = 55·4 when 0·5 *M* urea solution is compared with distilled water.

In the same way, the drops would change their length 34·4 (87·4 − 53·0) when 0·25 *M* urea solution is compared with water, and 21·0 tenths of a scale division when 0·5 *M* solution is compared with 0·25 *M* solution.

But the change of drops actually observed at 35° for 24 hours was far greater than these calculated values (Table IV (*e*), (*f*), (*g*)).

The amount of change of the drops was 249, 148·5, 128·8 (mean values) respectively in the cases of 0·5 *M* : water; 0·25 *M* : water; and 0·5 *M* : 0·25 *M* (Table IV (*e*), (*f*), (*g*)).

The amount of change in the drops when compared at the same temperature for the same length of time was thus found to be about 5–6 times greater than the value calculated from the velocity of evaporation of each solution. It must be assumed therefore that the proportion of change due to the vapour pressure is only $\frac{1}{6}$–$\frac{1}{7}$ of the entire change.

This ratio was also obtained in the case of boric acid solutions; when, however, lactose solution was investigated in this way, the ratio was a little smaller. The mean amount of evaporation from a single drop was respectively 0·5 *M* = 30, 0·25 *M* = 50, 0·1 *M* = 70·2, while the mean change of drops according to Barger's method was respectively

0·5*M* : water	175
0·25*M* : water	90
0·1*M* : water	68
0·5*M* : 0·25*M*	68
0·5*M* : 0·1*M*	112
0·25*M* : 0·1*M*	53

Table IV (*e*)

The amount of volume change of drops in Barger's method, at 35° C., in the first 24 hours, 0·5*M* urea compared with water.

No. of tube	Diameter of tube	1 (urea)	2 (water)	3 (urea)	4 (water)	5 (urea)
1	470	635 + 246 (1100)	674 – 224 (1200)	593 + 207 (1210)	676 – 231 (1095)	781 + 260 (1100)
2	415	614 + 271 (1120)	586 – 201 (1180)	707 + 213 (1150)	770 – 220 (1200)	680 + 270 (1280)
3	575	620 + 265 (1085)	640 – 235 (1060)	914 + 238 (990)	680 – 252 (1170)	663 + 220 (1300)

The mean amount of volume change = 249 tenths of a scale division.

(*f*)

The amount of volume change of drops in Barger's method, at 35° C., in the first 24 hours, 0·25*M* urea compared with water.

No. of tube	Diameter of tube	1 (urea)	2 (water)	3 (urea)	4 (water)	5 (urea)
1	470	484 + 136 (1090)	590 – 129 (1070)	425 + 160 (830)	520 – 150 (1330)	610 + 150
2	510	450 + 160 (940)	462 – 147 (1400)	365 + 130 (1250)	520 – 133 (1150)	431 + 159
3	440	465 + 148 (985)	437 – 153 (1210)	442 + 138 (1345)	415 – 129 (980)	514 + 146

The mean amount of volume change = 148·5 tenths of a scale division.

(*g*)

The amount of volume change of drops in Barger's method, at 35° C., in the first 24 hours, 0·5*M* compared with 0·25*M* urea.

No. of tube	Diameter of tube	1 (0·5*M*)	2 (0·25*M*)	3 (0·5*M*)	4 (0·25*M*)	5 (0·5*M*)
1	490	585 + 132 (925)	550 – 95 (1150)	520 + 114 (840)	471 – 124 (870)	720 + 110 (1200)
2	425	683 + 121 (1080)	540 – 102 (845)	522 + 140 (705)	525 – 150 (624)	676 + 140 (1190)
3	415	593 + 128 (985)	564 – 115 (725)	492 + 127 (965)	530 – 141 (910)	588 + 148 (1080)

The mean amount of volume change = 128·8 tenths of a scale division.

(The numbers in brackets denote the lengths of the air spaces between the drops.)

The ratio between the calculated value and the actual amount for lactose solutions was respectively

0·5*M* : water	3·0 : 1
0·25*M* : water	2·4 : 1
0·1*M* : water	4·0 : 1
0·5*M* : 0·25*M*	3·5 : 1
0·5*M* : 0·1*M*	2·8 : 1
0·25*M* : 0·1*M*	2·6 : 1

This small ratio probably does not indicate a greater part played by the vapour pressure here than in the case of urea or boric acid solutions, because

the evaporation of the single drops also takes place with a smaller speed than in the case of these latter solutions.

The probable explanation for this smaller ratio in the former case is that the molecules of sugar have a greater weight than those of urea and boric acid, hence the speed with which the former move in the solution is smaller than that of the latter. Now, in the case of the single drops, the surface of the drops becomes concentrated when evaporation proceeds, and this is also the case with the drops of weaker solutions in Barger's method, while the surface of the drops of stronger solutions becomes diluted by the entering of solvent.

It will take some time before these concentrated or diluted surface layers of the drops mix completely with the interior portions, causing the whole drop to become homogeneous. The slower the molecule moves the more time this diffusion will take, and thus the surface layer of the drops containing heavier molecules will remain longer in a concentrated or diluted condition than the surface of drops with lighter molecules.

This will result in slower evaporation of a solution with large molecules (this does not necessarily mean that the vapour pressure of such a solution is smaller). The smaller amount of evaporation, obtained in the single drop experiment, must have been due to this process. On the other hand, the volume-change of drops in Barger's method will be lessened by the slow mixing of the surface layer with the interior.

Thus the calculated amount and that actually observed are both influenced by the slowness of the movement of the molecules, and their ratio is affected doubly by this cause.

These ratios did not change when the same experiment was performed at 0°, although the amount of evaporation as well as the amount of the drop-change in Barger's method was reduced to $\frac{1}{6}$–$\frac{1}{7}$ of the value measured at 35°. It is of course evident that these ratios will change according to the length of the intervals between the drops. The part played by the streaming of solvent through the contact film of the drops will become smaller with the increase of the distance between them, so that the change of drops will be produced finally only by the difference of vapour pressure when some limit of distance has been exceeded. But under ordinary conditions, as directed by Barger (2–3 mm.), the amount of change ascribable to vapour pressure seems to be very small, and his method becomes quite unpracticable if the distance between the drops is so great that the drop-change is produced solely by the difference of vapour pressure.

The next question to be solved is what is the other factor which causes the volume-change of drops in this method. This cannot be other than the molecular and ionic attraction of solute for solvent, namely the force which is known also to give rise to the phenomenon of osmotic pressure. In this connection it seems to me that the "bombardment" theory of osmotic pressure, as represented in the text-books of Nernst, Lewis, Hedin, etc., is

less acceptable than van't Hoff's conception, particularly as explained more precisely by Mathews [1916].

"Osmosis" should be explained as a phenomenon of the wandering of the solvent molecules from a point where a weaker attractive power of solute molecules exists towards a point where a stronger attractive power is exerted. It does not matter whether there is a semipermeable membrane or not.

The force which produces the change of drops in Barger's method should thus be considered as the molecular-ionic attraction of solute for solvent.

Moreover, the change of the drops themselves seems to be due both to osmosis and to distillation, which are both caused by the same internal energy of solution, namely, the molecular-ionic attraction.

Summary.

The principle upon which Barger's microscopical method of determining molecular weights is based has been studied, and it has been proved that the force which effects the volume-change of the drops is not simply the vapour pressure, but also the osmosis through the thin film of liquid between the drops. This latter factor plays the greater part in producing the change.

If the length of the intervals between the drops is 2–3 mm., $\frac{5}{6}$ to $\frac{6}{7}$ of the volume-change of the drops is attributable to osmosis.

Further, as osmosis as well as the lowering of vapour pressure are equally the results of one and the same internal energy of solution, that is, the molecular and ionic attraction of solute for solvent, which is of the same magnitude for all kinds of molecules and ions in the same solvent at the same temperature, it is possible to measure molecular weights or the degree of association or dissociation by investigating the volume-change of drops which are brought in contact with drops of standard solutions of known molecular concentrations.

In conclusion I wish to express my heartiest thanks to Dr C. J. Martin, the Director of the Lister Institute, who has kindly supplied me with material and apparatus. Prof. G. Barger, who has read this paper in manuscript, informs me that he is in general agreement with my conclusions. He has frequently observed the effect of Exp. III, when using organic solvents. Like myself in my experiments with filter papers he has entirely failed in repeated attempts to bring about a regular isothermic distillation in a closed space, when there was no film of liquid between two solutions differing only slightly in vapour pressure.

REFERENCES.

Barger (1904). *J. Chem. Soc.* **85**, 286.
Mathews (1916). *Physiological Chemistry*, 2nd ed., William Wood & Co.

XV. THE FORMATION OF FERROUS SULPHIDE IN EGGS DURING COOKING.

By CHARLES KENNETH TINKLER and MARION CROSSLAND SOAR.

From the Chemical Laboratory, Household and Social Science Department, King's College for Women.

(*Received February 27th, 1920.*)

It is a matter of common experience that on prolonged cooking of an egg in its shell (*e.g.* when it is immersed in boiling water for 15 minutes or longer) a greenish black coloration is produced on the surface of the yolk.

It is also well known that if a so-called "hard-boiled egg" be immersed in cold water immediately after cooking, the green colour is either not apparent at all, or is much less marked than is the case when the egg is allowed to cool slowly.

In view of the fact that this coloration is only on the *surface* of the yolk, it would appear that in its production some chemical action takes place, either between some constituent of the membrane which surrounds the yolk and some constituent of the yolk itself, or that some substance which is produced from the egg white, and which can penetrate this membrane, interacts with some constituent of the yolk.

The Nature of the Coloured Substance.

It appeared probable at the outset of the investigation that the greenish black coloration was due to ferrous sulphide in a fine state of division, and the various experiments which have been made seem to confirm this view.

Thus the coloured substance is readily soluble in dilute acids, the solution so obtained giving positive reactions for iron and evolving hydrogen sulphide on warming, whereas cooked egg yolk showing no greenish colour does not give these reactions. Moreover, the coloured egg yolk is decolorised on prolonged exposure to air or on treatment with hydrogen peroxide. This is also the case with the greenish coloured precipitate of ferrous sulphide when, for example, this substance is precipitated in a fine state of division on a piece of filter paper by the interaction of a solution of ferrous sulphate and ammonium sulphide.

It is well known that egg white on prolonged heating evolves considerable quantities of hydrogen sulphide and that the yolk of egg contains iron, probably in combination with proteins. The amount of iron in egg yolk, according to Sherman [1914], is 0·0085 % by weight. A solution of ferrous

ammonium sulphate containing iron equivalent to that in egg yolk was made up and portions of the solution poured on to filter papers. On the addition of colourless ammonium sulphide to these filter papers, colorations due to ferrous sulphide were obtained, which were almost identical with those obtained on the yolks of eggs when unbroken eggs are immersed for 15 minutes or more in boiling water.

Pollacci [1904] has investigated the losses of sulphur which take place on heating yolk and white of egg separately, and also the effect of keeping uncooked whole eggs for 24 hours in an atmosphere of hydrogen sulphide. The chocolate brown colour produced in the yolk of the uncooked egg in this case is said to be due to the formation of iron sulphide.

The Effect of Prolonged Heating on Yolk and White of Egg separately.

Evidence that the green coloration was due to interaction between constituents of both the yolk and the white was obtained in the following way. Two yolks in their membranes, separated completely from egg white and washed with distilled water, were heated in boiling water for 20 minutes. One was then cooled quickly by washing in a stream of cold water, and the other was allowed to cool slowly. In neither case was the slightest green colour observed, but on placing portions of these yolks in a gas jar containing hydrogen sulphide to which a few drops of ammonia had been added, green colours were obtained both in the portions still covered with membrane and on those taken from the inside of the yolk.

It was found that the green colour was produced only very slowly by the action of hydrogen sulphide alone, but was immediately formed on the addition of ammonia.

Yolk of egg, separated from egg white, was also heated alone in a test tube for 90 minutes in boiling water, and even in this case no green coloration was apparent. Cooked egg white treated in the same way with hydrogen sulphide and ammonia gives no coloration until a considerable time has elapsed, and even then the colour produced is very faint. It should be noted in this connection that the iron content of egg white (0·0001 %) is only about one-eighty-fifth that of egg yolk [Sherman, 1914]. According to Pollacci [1904], egg white is free from iron.

From the results of these experiments it may be concluded that some constituent of the white of egg plays a part in the formation of the green layer of ferrous sulphide. Additional evidence that the yolk alone does not give rise to both constituents necessary for the formation of the ferrous sulphide is afforded by the fact that the latter is only produced as a thin layer on the surface of the yolk. If both the iron and sulphur were provided by the yolk, the green coloration would probably extend through the whole of the yolk, diminishing in intensity on approaching the centre, owing to the fact that the temperature at the centre of the egg does not rise as rapidly on cooking as that of the outside of the yolk.

It can also be shown that whereas neither white nor yolk of egg on heating alone shows any green coloration, when heated in contact with one another the characteristic green coloration is produced (see below).

Experiments relating to the Membrane round the Yolk.

In order to determine whether or not any constituent of the membrane round the yolk takes any part in the reaction, an experiment was made as follows. Egg white was heated in a test tube until coagulated, and egg yolk, free from membrane, was then poured into the test tube to form a layer above the white. On heating the test tube for about 15 minutes in boiling water, a green ring was obtained at the junction of the yolk and white.

On examining a "hard-boiled" egg, the colourless yolk membrane can be seen adhering to the inside of the white of the egg, and does not become absorbed by the yolk as appears at first.

It would seem, therefore, that the membrane round the yolk does not play any part in the formation of the green coloration. It must, however, be permeable to hydrogen sulphide.

The Effect of Time of Heating on the Coloration obtained.

Two eggs were heated in boiling water for 15 minutes and two for 30 minutes. In both cases one of the eggs was cooled down quickly by removing the shell and placing in a stream of running water, while the other was allowed to cool with the water in which it had been heated.

In the case of the eggs heated for 15 minutes practically no green colour was obtained on the yolk of the egg which was cooled quickly, whereas a slight green colour was apparent on the other.

In the case of the eggs heated for 30 minutes, very little difference was apparent in the amount of green colour developed, whether the egg was cooled quickly or allowed to cool with the water; but a much more intense coloration was produced round the yolks of these eggs than round the yolks of those heated only for 15 minutes.

It appears, therefore, as would be expected, that the amount of green coloration depends upon the length of time the egg is heated, and that the effect of the cold water in preventing the formation of the green colour is simply that, on cooling the egg quickly, the preliminary decomposition necessary for the formation of ferrous sulphide is checked. The act of placing a hard-boiled egg in cold water does not therefore lead to the *removal* of the green coloration already formed, but prevents its formation.

This view is supported by the following observation. Two eggs were heated in boiling water for 15 minutes, the shell of one was removed at once and the white of the egg removed. No green colour was observed. The white was then replaced round the yolk and secured with cotton to prevent the access of air. The egg was allowed to cool slowly, and when cold, the white of the egg was again removed. The green coloration was then apparent.

The other egg was allowed to cool in the water in which it had been cooked and, on examination when cold, was found to show the usual green coloration round the yolk. In addition to the effect of the cold water in checking the decomposition of the egg white, the rapid cooling on the outside of the egg will lead to the diffusion of the hydrogen sulphide from the white away from the yolk, and in this way also diminish the amount of ferrous sulphide formed.

In order to investigate further the effect of prolonged heating on the formation of the colour, two eggs were heated in boiling water, one for two hours, when still only a thin film of green round the yolk was apparent, and the other for seven hours. In the latter case the colour of the white after cooking was buff and the green layer was thicker than in the previous case, especially on one side where its thickness was about one millimetre.

Subsequent experiments showed that if the yolk of an egg after cooking is not surrounded by a layer of white of uniform thickness, the thickness of the layer of ferrous sulphide is greater where the yolk has been surrounded by a greater thickness of white.

The object of heating one of the eggs for seven hours was also to ascertain, if possible, whether the hydrogen sulphide from the white could penetrate the thin film of ferrous sulphide at first formed, and produce in the yolk layers of ferrous sulphide as in the Liesegang phenomenon. No such layers were, however, apparent, either in the yolk of this egg cooked in boiling water for seven hours, or in an egg yolk which, completely surrounded by its membrane, washed free from adhering white, and cooked in boiling water for 20 minutes, was then placed in a gas jar with hydrogen sulphide and ammonia.

It was found that if an uncooked egg is kept for a day in an atmosphere of hydrogen sulphide, as in Pollacci's experiments, and then heated in boiling water for 20 minutes, the greenish black coloration due to ferrous sulphide extends throughout the whole of the yolk.

The Effect of the Age of the Egg upon the Amount of Green Coloration.

It was thought that the age of the egg might determine to a considerable extent the amount of green coloration formed on prolonged cooking. To ascertain if this were so, three eggs, (1) an egg laid one day previously, (2) an egg laid five weeks previously, (3) an egg laid six weeks previously, were immersed in boiling water for 20 minutes and allowed to cool in the water. The yolks were then separated from the whites and treated in exactly the same way, each being immersed in dilute iron-free sulphuric acid. After ten minutes the acid liquid was filtered and the solutions diluted to the same volume in each case. There was certainly a difference in the amount of iron contained in the solutions; the extract from the yolk of the fresh egg containing least iron, and that from the six week old egg most, but the difference was not very great. This was in accordance with the appearance of the yolks before treatment with the acid. Only slightly more green coloration was

apparent in the case of the egg laid six weeks previously, than in the case of the one day egg.

The Liberation of Hydrogen Sulphide from Egg White and Yolk.

Although the sulphur content of the yolk of an egg—0·157 %—is not very much less than that of the white—0·196 % [Sherman 1914]—this element is evidently in less stable combination in the latter than in the former. On heating white and yolk of egg separately in a test tube immersed in boiling water, hydrogen sulphide is evolved (shown by its action upon lead acetate paper) from the white after about three and a half minutes, while no trace of the gas is obtained from the yolk in this time.

An approximate comparison of the amounts of hydrogen sulphide obtained on distilling equal weights of egg white and yolk (1 g.) with water (400 cc.) was obtained by the addition of standard lead acetate solution to the distillates, and comparing the colours obtained with those formed with known amounts of the lead acetate solution to which hydrogen sulphide solution had been added.

In the case of the egg white the first 100 cc. of the distillate was matched by 2·5 cc. of standard lead acetate solution (1 cc. = 0·0001 g. Pb), whereas a similar volume of the distillate from the egg yolk was free from hydrogen sulphide.

On continuing the distillation 2 cc. of the standard lead solution were required for the next 100 cc. of the distillate from the egg white and 0·7 cc. from a similar volume of distillate from the yolk.

Sulphuretted hydrogen is thus more readily obtained from egg white than from egg yolk. [See also results obtained by Pollacci, 1904.]

The Reactions of Egg White and Yolk towards Litmus.

Uncooked egg white has an alkaline reaction, and uncooked egg yolk an acid reaction, towards litmus. It was at first thought possible that ammonia, or a substituted ammonia, might be produced by the decomposition of the egg white. With the hydrogen sulphide this ammonia or amine might pass through the membrane, and by neutralising the acid of the yolk facilitate the production of ferrous sulphide. Small quantities of ammonia and substituted ammonia are produced both from the white and yolk on distillation with water, but this ammonia is perhaps not necessary for the production of the ferrous sulphide, as it was noticed in the course of the experiments that the yolk of egg, which is originally acid to litmus, becomes alkaline on heating.

On heating a mixture of egg yolk and water containing blue litmus the indicator is seen to change colour at approximately 70°.

When an egg is heated in boiling water in the ordinary process of "boiling" for three to five minutes, the temperature of the yolk probably does not reach 70°, so that if hydrogen sulphide were present it might possibly not react immediately with the yolk to form ferrous sulphide, owing to the acid

reaction of the yolk at a temperature below 70°. On prolonged heating, however, the yolk will become alkaline and the conditions for the formation of ferrous sulphide will be favourable.

The Formation of Ferrous Sulphide in preserved and dried Eggs.

Eggs which have been preserved in water glass appear to behave normally with regard to the formation of ferrous sulphide on cooking. Thus two eggs which had been preserved for ten months in water glass were boiled for 20 minutes. One was cooled quickly and the other allowed to cool slowly. In the case of the former no formation of ferrous sulphide was apparent, but in the case of the latter the usual appearance of the substance on the surface of the yolk was noted.

Eggs which have been dried appear to be altered in some way which largely prevents the formation of iron sulphide on prolonged cooking. Thus if the white and yolk of an egg be beaten together and some of the mixture heated in a test tube in boiling water for 20 minutes, a slight green colour is apparent, whereas if the beaten egg is dried in a vacuum desiccator and then mixed with water immediately after drying, only a very faint coloration is observed on cooking. If the dried egg is kept for some time, the formation of ferrous sulphide on cooking does not take place. Some commercial preparations of dried eggs also failed to show the formation of ferrous sulphide on cooking.

SUMMARY.

The greenish black coloration observed on the surface of the yolk of a "hard-boiled" egg appears to be due to the formation of ferrous sulphide, the hydrogen sulphide necessary for the formation of the substance being produced by the decomposition of a sulphur compound of the egg white.

The non-formation of the coloration when a "hard-boiled" egg is placed in cold water immediately after cooking is due to the checking of the decomposition owing to cooling.

REFERENCES.

Pollacci (1904). *Gazzetta*, 34 (i), 278.
Sherman (1914). *Food Products* (Macmillan).

XVI. ON THE PRESENCE OF AMYLASE IN MILK AND CHEESE.

By MASAYOSHI SATO.

From the Columbia University, New York.

(*Received February 28th, 1920.*)

There has been little investigation regarding enzymes in raw milk, especially amylase. In 1883, according to the literature, Béchamp [1883] isolated from human milk an enzyme which liquefies starch and converts it into sugar, and named it galactozymase. He claimed that the enzyme was not present in cow's milk. His work was recognised by Bouchut [1885], Moro [1898], Biolchini and Luzzati [1902], Hippius [1905], and Spolverini [1902]; the last named found very little amylase in dog's, ass's and human milk and none in cow's and goat's milk.

Zeitscheck [1904] found evidence of the existence of slight amylolytic activity in all the samples of human, ass's, horse's, goat's, buffalo's and cow's milk examined by him. Spontagh [1905] also proved the presence of amylase in cow's and other milk which he examined.

Wohlgemuth and Strich [1910] were unable to obtain any evidence of the presence of amylase in cow's milk but found it in dog's, rabbit's and guinea-pig's milk, dog's milk containing most and guinea-pig's the least.

Koning [1907] recognised, on all occasions, evidence of amylase in cow's milk; thus he found that about 15–20 mg. of starch were decomposed by 100 cc. of milk in half-an-hour at 37°; and Giffhorn [1909] also found that 100 cc. of milk will decompose from 10–25 mg. of starch in one half-hour at 37°. Lane-Claypon [1913] carried out investigations upon the amylase content of sterile cow's milk collected by means of a collecting tube. Amylase was found in each experiment, but the amount present was so small that the 10 cc. of milk used were able to hydrolyse only from 0·001–0·002 g. of starch in three hours at 37°.

Regarding the activity of amylase in milk Koning found that the optimum temperature was 45° and that it was destroyed by heating the milk for 45 minutes at 68°, but Giffhorn found that the enzyme action was destroyed by heating at 65° for 30 minutes.

In regard to the source of this ferment in cow's milk there are some diversities of opinion among investigators.

According to Spolverini this ferment was obtained from cow's and goat's milk, which did not generally contain amylase, when the animals were fed with fodder containing a large quantity of starch, such as malt, and in such cases he was of opinion that a part of the amylase of milk may be derived from the udder and illustrated it by Dupony's experiment in which he proved that the amount of amylase was increased in the blood, liver and kidney by feeding with carbohydrates. But this opinion was denied by van der Velde and Landtscher [1909] who got negative results by the same experiment.

Koning showed that amylase was present in milk of animals with mastitis and that in normal milk the first and middle milks were richer than the strippings and also that certain bacteria were capable of producing amylase, but he believed that the amylolytic action obtained by him with cow's milk was not due to contamination by bacteria. Giffhorn also believed that amylase is increased considerably in milk by the pathological condition of the udders.

Wohlgemuth and Strich insisted that a part of the amylase of milk may be derived from the blood, but the milk generally contains at least 100 times as much amylase as the blood, the great part of this being a product of the activity of the mammary glands. Grimmer [1913] obtained evidence of its presence by his experiment on the gland tissue of animals of which the milk showed only little or no trace of amylase. Upon this experimental basis he suggested that amylase in milk was the product of the mammary gland.

As an application of the detection of the existence of amylase in milk, Koning carried out an experiment with the object of discovering whether milk has been boiled before being sold, and also stated that the presence of milk from a mastitis cow can be discovered by testing for this ferment. The former is generally used as a supplementary test to detect whether milk has been boiled or not, but the latter observation is not, at present, recognised as a reliable aid in the diagnosis of mastitis.

As to the degree to which starch is broken down by the amylase in milk, Moro [1902] (working with human milk) believed that some of it was converted into dextrin and maltose but that most of it remained in the stage of dextrin. The difficulty of estimating the terminal point of this reaction in milk is, however, very great, since it is almost impossible to detect the formation of minute quantities of maltose.

In short, at the present time nothing definite is known regarding the existence, origin and biological action of this enzyme in cow's milk. We also lack knowledge of any literature on amylase in cheese.

In the present investigation, as the first step, we examined raw milk for the existence and activity of amylase, and likewise the water-extract of fat-free cheese, determining in each case the power of liquefying potato- and also rice-starch, to see what difference if any exists in the effect of the amylase of raw milk and of cheese in liquefying these two starches.

Materials.

Milk and cheese.—The grade *A* raw certified milk and American Cheddar cheese used in the experiment were supplied by the Sheffield Farm Company in New York city.

Distilled water.—The distilled water which was used in the experiment was twice redistilled, first from alkaline permanganate solution into a reservoir whence it siphoned into a second flask from which it was again distilled. Phosphoric acid was added to this portion in order to retain any ammonia that might have come from the first distillation. Tin condensers were used and the final distillate was caught in "Nonsol" bottles, contact with the air being avoided as far as possible.

Substrates.—The substrates used in the experiment were eight samples, five of potato- and three of rice-starch, prepared by the following methods:

No. 1. Commercial potato-starch as purchased and further purified by repeated washing with distilled water.

No. 2. Potato-starch was prepared in the laboratory from mature August potatoes, care being taken to prevent contamination by organisms, dust, fumes, or water containing electrolytes.

No. 3. Potato-starch was prepared from potato-starch No. 2 by washing with 0·3 % NaOH.

No. 4. Lintner soluble potato-starch purchased from Merck and further purified with distilled water.

No. 5. Soluble potato-starch prepared from potato-starch No. 2 in the laboratory by Brown and Morris' method of treatment with dilute hydrochloric acid.

No. 6. Rice-starch prepared in the laboratory from the commercial product in the same way as potato-starch No. 2.

No. 7. Rice-starch prepared from rice-starch No. 6 by washing with 0·3 % NaOH.

No. 8. Soluble rice-starch prepared from starch No. 6 in the laboratory in the same way as the soluble potato-starch (No. 5).

Note:—For convenience, the different preparations of starch are designated by the following figures:

P.S. I	= Starch No. 1	P.S. V	= Starch No. 5
P.S. II	= „ „ 2	R.S. I	= „ „ 6
P.S. III	= „ „ 3	R.S. II	= „ „ 7
P.S. IV	= „ „ 4	R.S. III	= „ „ 8

Apparatus.

Thermostat.—The digestion of starch by the amylase was carried out in a bath which contained water kept at 40° by means of carbon filament lamps. The water in the bath was stirred constantly to ensure an even temperature throughout the experiment.

Glassware.—All glassware used in the experiment was treated to avoid any condition which could effect enzyme action.

Analysis of the starch.

The moisture, amylose and acidity of the various samples of starch were determined by means of the following methods:

Moisture. From 0·2 to 1·0 g. was dried in an air-oven at 100°–110° for four hours, allowed to cool in a desiccator over sulphuric acid for half-an-hour, and weighed.

Amylose. Amylose was determined by hydrolysing to glucose and estimating the glucose by Fehling's volumetric method.

Acidity. Acidity was determined by titration of the starch paste and solution with 0·001 *N* sodium hydroxide or sulphuric acid, using rosolic acid as indicator.

The results obtained are given in Table I.

Table I.

Substance	Moisture %	Amylose %	Acidity (cc. of *N*/1000 acid or alkali, per 1 g. amylose)
P.S. I ...	14·64	84·59	9·21 (H_2SO_4)
P.S. II ...	13·79	85·88	7·19 „
P.S. III ...	16·65	89·29	14·22 „
P.S. IV ...	9·73	90·00	3·83 (NaOH)
P.S. V ...	13·84	85·55	2·44 (H_2SO_4)
R.S. I ...	8·43	91·09	3·49 „
R.S. II ...	11·69	86·54	7·89 „
R.S. III ...	9·26	90·36	4·21 „

Preparation of potato- and rice-starch paste and solution.

The amount of starch equivalent to 1 g. of amylose was placed in an Erlenmeyer flask and 80 cc. distilled water added. The liquid was then heated slowly, with constant shaking, until it boiled, and the boiling was continued during three minutes. The flask was then cooled and the alkalinity or acidity of the starch was corrected by adding to each starch paste or solution sufficient *N*/1000 H_2SO_4 or *N*/1000 NaOH to make it exactly neutral, after which the volume was made up to 100 cc. with distilled water and the sample kept in an ice-box at 7°–8° until used.

The amylase activity of raw milk.

As soon as the milk sample reached the laboratory, it was well mixed and, after the estimation of its acidity with *N*/10 NaOH, was divided into two portions, one of which was kept raw, the other pasteurised by heating for 30 minutes at about 80°. The former was used for the enzyme examination, and the latter as control.

The following procedure based upon the method of Koning with slight modification was used.

Two series of test-tubes were very carefully cleaned and dried, placed on the test-tube-stand and the whole set in a bath of ice-water. Then known

quantities of starch paste or solution were carefully measured in increasing amounts, *e.g.* 0·001 cc., 0·002, 0·003, ..., into each of the tubes by means of a long 1 cc. pipette (divided into 1/100 cc.) reaching to the bottom of the test-tube, thus avoiding the lodging of any of the solution on the side of the tube.

10 cc. of raw or pasteurised milk, which had been kept in the ice-box at 7°–8°, were very accurately measured and introduced into each tube and agitated to ensure a perfect mixture.

The test-tube-stand was transferred from the ice-water to the thermostat and after various lengths of time was taken out and placed in the ice-water to stop the action. After a few minutes exactly 1 cc. of iodine solution (1 g. iodine, 2 g. potassium iodide + 300 cc. water) was added to each tube, and the contents thoroughly mixed. The tube in which the blue or violet colour had entirely disappeared, giving place either to red, orange or yellow, was noted and the power of the enzyme was calculated as the number of grams of starch which is digested to this stage in a given time by 100 cc. of raw milk.

Results.

The quantity of starch decomposed by nine samples of milk in half-an-hour and by four samples in 24 hours[1] is given in Table II.

In the control experiment with pasteurised milk it was found that 10 cc. of the milk was not able to hydrolyse more than 0·002 g. of starch (0·001 g. of starch in 10 cc. of milk cannot be detected by this method).

Table II.

(1) The quantity of starch decomposed by 100 cc. raw milk in 30 minutes at 40°.

	P.S. I	P.S. II	P.S. III	P.S. IV	P.S. V	R.S. I	R.S. II	R.S. III
	g.	g.	g.	g.	g.	g.	g.	g.
Average of 9 experiments	0·0092	0·0093	0·0096	0·0151	0·0154	0·009	0·010	0·0081
Maximum	0·011	0·011	0·011	0·021	0·021	0·010	0·012	0·010
Minimum	0·005	0·005	0·005	0·012	0·012	0·005	0·005	0·004

(2) The quantity of starch decomposed by 100 cc. of raw milk in 24 hours at 40°.

Average of 4 experiments	0·0233	0·0235	0·0245	0·034	0·035	0·0233	0·0243	0·023
Maximum	0·026	0·027	0·027	0·035	0·036	0·025	0·026	0·025
Minimum	0·022	0·022	0·022	0·034	0·034	0·022	0·023	0·022

From Table II it will be seen that the eight preparations of starch employed can be arranged in the following order, according to the readiness with which they were digested:

Digestion for 30 minutes.

P.S. V; P.S. IV; R.S. II; P.S. III; P.S. II; P.S. I; R.S. I; R.S. III.

Digestion for 24 hours.

P.S. V; P.S. IV; P.S. III; R.S. II; P.S. II; (P.S. I; R.S. I); R.S. III.

In all cases, preparation P.S. V was decomposed most readily, and the digestion of R.S. III was the slowest. Preparation R.S. II which was washed with alkali was decomposed somewhat faster than P.S. III which was treated

[1] 0·3 cc. of toluene was added in these cases per 10 cc. of milk as antiseptic.

in the same way, in the course of digestion during 30 minutes. But, on the contrary, the digestion of the former preparation was rather less than that of the latter in the course of 24 hours.

We undertook the next experiment, choosing three preparations. In order to make these relationships clearer the digestion of three of the preparations P.S. III, P.S. V and R.S. II was carried out for 30 minutes, 1, 3, 5, 7, and 24 hours respectively; the results are given in Table III.

0·3 cc. of toluene was used in each experiment per 10 cc. of milk.

Table III.

The quantity of starch decomposed by 100 cc. of raw milk.

	P.S. III g.	P.S. V g.	R.S. II g.	Acidity of milk $\frac{N}{10}$ alkali per 100 cc. cc.
In 30 minutes.				
Average of 8 experiments	0·0074	0·0164	0·0083	23·53
Maximum	0·010	0·018	0·010	23·92
Minimum	0·005	0·015	0·007	22·88
In 1 hour.				
Average of 8 experiments	0·0094	0·0244	0·0099	
Maximum	0·012	0·027	0·012	
Minimum	0·007	0·022	0·008	
In 3 hours.				
Average of 8 experiments	0·0123	0·0296	0·0126	
Maximum	0·016	0·031	0·016	
Minimum	0·009	0·028	0·010	
In 5 hours.				
Average of 8 experiments	0·0144	0·033	0·0145	
Maximum	0·019	0·035	0·019	
Minimum	0·010	0·031	0·010	
In 7 hours.				
Average of 8 experiments	0·015	0·0327	0·0151	
Maximum	0·020	0·034	0·020	
Minimum	0·010	0·032	0·020	
In 24 hours.				
Average of 8 experiments	0·0182	0·0363	0·0165	
Maximum	0·025	0·037	0·023	
Minimum	0·011	0·035	0·010	

As shown in Table III, the amount decomposed during 30 minutes was the largest in the case of P.S. V, the order being P.S. V—R.S. II—P.S. III.

This order was also maintained for the digestions of one, three, five and seven hours respectively, but was disturbed in the digestion of 24 hours by P.S. III which now came ahead of R.S. II, the order being accordingly changed to P.S. V—P.S. III—R.S. II. P.S. V was always decomposed most readily. Thus we could ascertain the fact that, at the beginning of digestion, rice-starch is decomposed a little faster than potato-starch, though the difference cannot be said to be very remarkable.

The presence of amylase in cheese.

Composition of the cheese.

The composition[1] of the samples of cheese used in this experiment was as follows (Table IV):

Table IV.

	Cheese sample I %	Cheese sample II %
Water	34·720	36·883
Fat	29·623	31·175
Protein	31·175	27·175
Lactic acid, etc.	1·450	1·262
Ash	3·032	3·504

Preparation of a water-extract of fat-free cheese.

The following method was used for the preparation of a water-extract of fat-free cheese.

The crusts of the cheese were removed with a knife and it was then well ground in a mortar and dehydrated with alcohol. The ground cheese was extracted with ether in a Soxhlet apparatus for about three days or longer, in order to remove the fat completely. The fat-free material was then spread on a porous plate, dried at the room temperature and ground to a fine powder. 10 g. of this material were transferred to a stoppered flask containing distilled water, kept cold (7°–8°), and allowed to stand in the ice-box with frequent shaking for 1½ or 24 hours. In the latter case 1 cc. of toluene was used per 50 cc. of water as antiseptic. The solution was then filtered by suction through a Buchner funnel, containing a thin mat of long-fibred asbestos, and made up to 500 cc. with distilled water.

Method of experiment.

The experiment was carried out in the same way as that with milk, 0·3 cc. of toluene being added for 10 cc. water-extract of fat-free cheese.

As the first step, the activities of the amylase of the 24 hours' water-extract with toluene and 1½ hours' water-extract without toluene were determined, using potato soluble starch as substrate.

The results are given in Table V.

Table V.

	1½ hours' water-extract (without toluene)		24 hours' water-extract (with toluene)	
Time	Exp. I g.	Exp. II g.	Exp. I g.	Exp. II g.
30 minutes	0·008	0·0095	0·003	0·006
1 hour	0·010	0·012	0·004	0·008

[1] The analyses were made by Richmond's method.

It will be seen from Table V that when the 24 hours' extract with toluene is compared with the 1½ hours' extract without antiseptics, the latter is found to be much more active than the former. Further experiments on this point were therefore considered unnecessary, and in studying amylase in cheese the 1½ hours' extract without addition of toluene was used throughout the investigation.

Results of experiments.

We investigated the digestion of the four kinds of starch P.S. III, P.S. V, R.S. II, R.S. III, comparing the quantity decomposed by cheese samples I and II during the lapse of 30 minutes, 1, 3, 5 and 24 hours respectively, and obtained the results shown in Tables VI, VII and VIII.

Table VI.

Cheese Sample I.

Grams of starch decomposed by 100 cc. of the water-extract of 2 g. fat-free cheese (=5·835 g. of original cheese at 40°).

	P.S. III g.	P.S. V g.	R.S. II g.	R.S. III g.	Acidity of water-extract of fat-free cheese *N*/10 alkali per 100 cc. cc.
In 30 minutes.					
Average of 19 experiments	0·0062	0·0073	0·0079	0·0054	6·60
Maximum	0·009	0·009	0·009	0·007	
Minimum	0·005	0·006	0·006	0·004	
In 1 hour.					
Average of 11 experiments	0·0081	0·0096	0·0108	0·0077	
Maximum	0·009	0·011	0·011	0·009	
Minimum	0·007	0·008	0·009	0·007	
In 3 hours.					
Average of 12 experiments	0·0141	0·019	0·0158	0·0146	
Maximum	0·015	0·021	0·018	0·016	
Minimum	0·013	0·018	0·014	0·013	
In 5 hours.					
Average of 5 experiments	0·0206	0·025	0·0202	0·0174	
Maximum	0·022	0·026	0·021	0·018	
Minimum	0·019	0·024	0·019	0·017	
In 24 hours.					
Average of 3 experiments	0·0303	0·036	0·0293	0·022	
Maximum	0·031	0·037	0·030	0·024	
Minimum	0·030	0·035	0·029	0·021	

Table VII.

Cheese Sample II.

Grams of starch decomposed by 100 cc. water-extract of 2 g. fat-free cheese (=6·519 g. of original cheese at 40°).

	P.S. III g.	P.S. V g.	R.S. II g.	R.S. III g.
In 30 minutes.				
Average of 7 experiments	0·0109	0·0126	0·0119	0·009
Maximum	0·012	0·013	0·013	0·010
Minimum	0·010	0·012	0·011	0·008
In 1 hour.				
Average of 7 experiments	0·0156	0·0174	0·0146	0·0124
Maximum	0·017	0·018	0·015	0·014
Minimum	0·015	0·016	0·014	0·012
In 3 hours.				
Average of 7 experiments	0·021	0·026	0·020	0·0189
Maximum	0·022	0·027	0·021	0·020
Minimum	0·020	0·025	0·019	0·018
In 5 hours.				
Average of 7 experiments	0·024	0·031	0·022	0·0209
Maximum	0·025	0·032	0·024	0·022
Minimum	0·023	0·030	0·022	0·019

Calculating the amount of starch decomposed by 100 g. of the original cheese from the averages given in Tables VI and VII, the following results were obtained (Table VIII):

Table VIII.

Cheese Sample I.

Time of digestion	P.S. III g.	P.S. V g.	R.S. II g.	R.S. III g.
30 mins.	0·1063	0·1251	0·1357	0·0925
1 hr.	0·1388	0·1645	0·1851	0·1320
3 hrs.	0·2416	0·3256	0·2691	0·2485
5 „	0·3530	0·4284	0·3464	0·2982
24 „	0·5193	0·6170	0·5021	0·3770
Cheese Sample II.				
30 mins.	0·1672	0·1902	0·1825	0·1381
1 hr.	0·2393	0·2669	0·2240	0·1902
3 hrs.	0·3068	0·3988	0·3068	0·2899
5 „	0·3682	0·4755	0·3528	0·3206

It will be observed in Tables VI, VII and VIII that the different samples of starch decomposed by the amylase of cheese can be arranged in the following order according to the amount decomposed.

Cheese Sample I.

30 mins.	...	...	...	R.S. II	P.S. V	P.S. III	R.S. III
1 hr.	...	...	...	R.S. II	P.S. V	P.S. III	R.S. III
3 hrs.	...	...	...	P.S. V	R.S. II	R.S. III	P.S. III
5 „	...	...	...	P.S. V	P.S. III	R.S. II	R.S. III
24 „	...	...	...	P.S. V	P.S. III	R.S. II	R.S. III

Cheese Sample II.

30 mins.	...	...	...	P.S. V	R.S. II	P.S. III	R.S. III
1 hr.	...	...	...	P.S. V	P.S. III	R.S. II	R.S. III
3 hrs.	...	...	...	P.S. V	R.S. II (P.S. III)		R.S. III
5 „	...	...	...	P.S. V	P.S. III	R.S. II	R.S. III

Thus we could confirm the same facts as were found with milk that the preparation P.S. V was decomposed most easily, that the digestion of R.S. III was the worst and that the digestion of R.S. II was better than that of P.S. III.

Summary.

1. The results of Spontagh, Zeitscheck, Wohlgemuth and Strich, Koning, Giffhorn, and Lane-Claypon regarding the presence of amylase in raw milk are confirmed.

2. For the quantity of soluble potato-starch decomposed by 100 cc. of raw milk, we find practically the same result as Koning and Giffhorn at half-an-hour, but more than was observed by Lane-Claypon in three hours.

3. As a result of our investigation, it appears that amylase is universally present in commercial Cheddar cheese, although the amount of this ferment varies within considerable limits.

4. Soluble potato-starch is digested more easily than potato- and rice-starch, both by milk and by the amylase of cheese.

5. Rice-starch seems to be more decomposed than potato-starch within a certain number of hours but the difference is so small as not to be distinct.

6. Soluble rice-starch is less decomposed than other starches in each experiment but it is uncertain what is responsible for this and further investigation is required.

I have to thank Prof. H. C. Sherman for helpful suggestion and interest throughout the work.

REFERENCES.

Béchamp (1883). *Compt. Rend.* **96**, 1508.
Biolchini and Luzzati (1902). *Arch. Kinderheilk.* **33**, 460.
Bouchut (1885). *Hyg. de la prem. Enfance*, 102.
Giffhorn (1909). *Dissertation, Hannover; Rev. gen. du Lait*, 1911, **8**, No. 22.
Grimmer (1913). *Biochem. Zeitsch.* **53**, 429.
Hippius (1905). *Jahrb. Kinderheilk.* **61**, 365.
Koning (1907). *Milchwirtsch. Zentr.* **3**, 41.
Lane-Claypon (1913). *Report to the Local Govt. Board*, N.S. **76**, 52.
Moro (1898). *Jahrb. Kinderheilk.* **47**, 342.
—— (1902). *Jahrb. Kinderheilk.* **56**, 362.
Spolverini (1902). *Rev. Hyg. Med. Enfant.* **1**, 252.
Spontagh (1905). *Jahrb. Kinderheilk.* **62**, 715.
van der Velde and Landtscher (1909). *Arch. Med. Enfant.* **6**, 408.
Wohlgemuth and Strich (1910). *Sitzungsber. Preuss. Akad. Wissensch.* **1**, 520.
Zeitscheck (1904). *Arch. ges. Physiol.* **104**, 539.

XVII. THE ANTISCORBUTIC REQUIREMENTS OF THE MONKEY.

By ARTHUR HARDEN and SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute.

(*Received February 27th, 1920.*)

The use of the monkey as an experimental animal in the investigation of scurvy and other deficiency diseases is becoming more and more extensive, owing chiefly to the fact that a great similarity exists between the clinical symptoms of the disease induced in these animals and the symptoms which occur in human subjects suffering from a similar disease contracted through the agency of faulty nutrition. Such experiments, however, are of a prolonged nature and several months must elapse before the results are known. This drawback coupled with the comparative scarcity and high price of the monkey makes this animal in many instances less suitable for experimental scurvy than the guinea-pig. The latter animal is plentiful in this country and results can be obtained with it within a few weeks. One can therefore consider these two animals as complementary in the technique of experimental scurvy, and it is accordingly desirable to establish some quantitative relationship as regards dose and time between them, so that results obtained with one can be translated if necessary into terms of the other.

It is now well established that guinea-pigs which are fed on a scorbutic diet of oats, bran and autoclaved milk manifest well-declared symptoms of the disease after about 15–21 days, although, as was shown by Zilva and Wells [1919], microscopical changes of the teeth may be present as early as 7–10 days after the commencement of the experiment. Our previous experiments [1919] with monkeys have shown that these animals usually manifest symptoms of scurvy after about two months. We have thus an approximate relationship as to the time taken by the two animals to develop scurvy when maintained on scorbutic diets.

It was the object of this investigation to find out the minimum dose of orange juice for monkeys. Orange juice was chosen because it is one of the most common antiscorbutics employed at present in infant feeding and on account of its sweet taste and comparatively low content in acids it can be administered without producing any digestive disturbances, both in human subjects and animals.

It is unlikely that orange juice has a constant antiscorbutic content, since this most probably varies with the variety of the fruit, time of the year and

other factors. This fact must be taken into consideration especially when, as in this case, the experiment is spread over a period of several months. It is at present impossible to standardise an antiscorbutic as regards its therapeutic potency by any chemical method, and the biological method now in vogue requires several weeks before a definite result is obtained and even then the degree of accuracy is not very great. Under these circumstances freshly expressed orange juice was considered to be the most suitable form of the antiscorbutic for our purpose. According to Chick and Rhodes [1918] the minimum dose of orange juice for a guinea-pig lies between $1\frac{1}{2}$ cc.–3 cc. per diem. Our own experience in this laboratory confirms this observation.

Five monkeys receiving 5 cc., 2 cc., 1 cc., 0·75 cc., 0·5 cc. of orange juice respectively were utilised in this experiment. The animals received a scorbutic diet consisting of rice, wheat germ, salt mixture, and butter. The daily ration, which was qualitatively and quantitatively adequate, was made up as follows:

Rice	300 g.
Wheat germ ...	50
Salt mixture ...	2
Butter	5

The orange juice was administered daily *per os* under strictly quantitative conditions, the smaller doses being previously diluted with sweetened water. The orange juice was pressed out fresh every day and endeavours were made to obtain oranges of the same variety throughout the duration of the experiment.

Doses of orange juice of 5 cc. and 2 cc. have proved to be sufficient to ward off scurvy. The monkeys receiving these doses throve well and gained in weight. The animals on the smaller quantities of juice developed scurvy which in some cases was severe enough to prove eventually fatal. The following are the protocols of the experiments.

Monkey No. 16. *Macacus rhesus*, male. Started on a scorbutic diet on May 13th, 1918 and received 0·5 cc. of orange juice daily. The weight of the animal, which at the commencement of the experiment was 2600 g., was maintained, apart from small fluctuations, until the end of June. After that period the animal commenced losing weight. Scorbutic symptoms became quite definite early in August, when purple and swollen gums were observed. The scorbutic condition developed gradually during the month of August. By the commencement of September the animal had lost the use of its hind limbs. At this stage it was at first intended to cure the monkey but after the administration of a single dose of antiscorbutic equivalent to about 20 cc. of lemon juice the plan was changed and the original daily dose of 0·5 cc. was resumed. The effect of the single high dose was very noticeable as the condition of the animal improved immensely. The pseudo-paralysis of the hind limbs disappeared but the gums and teeth did not clear up. The scurvy, however, soon became severe again and in spite of the continued daily dose

of 0·5 cc. of orange juice the animal declined and died on October 21st, 1918. At the post mortem examination very severe scurvy was found.

Monkey No. 24. *Macacus rhesus*, male. Experiment started July 29th, 1918. Dose 0·75 cc. of orange juice. The initial weight of this animal was 2900 g. Even in the early stages of the experiment the animal showed a slow loss in weight. This decline became very rapid at the end of October and lasted to the end of the experiment. As early as the middle of September the animal showed a tendency to bleed from the gums when handled but no more definite symptoms of scurvy were discernible at this stage. Spongy and purple gums however soon developed and by October 21st pseudo-paralysis of the hind limbs was recorded. The animal declined and died on November 22nd, 1918. At the post mortem examination severe scurvy was found.

Monkey No. 25. *Macacus rhesus*, male. Placed on scorbutic diet on August 26th, 1918. Dose 1 cc. of orange juice. Initial weight 2160 g. This monkey increased in weight until November 9th, 1918 when it reached 2600 g. After that time it commenced to lose slowly in weight. Early in December 1918 the animal developed spongy gums and its incisors became loose. At the same time it lost its liveliness and became dejected. It could however move its limbs without discomfort. As the mild scurvy could not be responsible for this condition it was concluded that the animal was suffering from some other disease. It was therefore decided to stop the experiment and the monkey was placed on a mixed diet on December 12th and a strong dose of an antiscorbutic was also administered. The scurvy cleared up but the animal died five days later. At the post mortem examination no scurvy was found and the cause of death could not be ascertained.

Monkey No. 15. *Macacus rhesus*, female. Placed on scorbutic diet February 16th, 1918. Dose 2 cc. of orange juice. This experiment lasted a year during which time the animal kept in excellent health and gained in weight regularly. The initial weight of the monkey was 3300 g. It weighed 4400 g. on February 19th, 1919 when it was chloroformed. At the post mortem examination the animal was found to be in excellent condition and no trace of scurvy was observed.

Monkey No. 14. *Macacus* (species not determined), male. Placed on scorbutic diet February 16th, 1918. Dose 5 cc. of orange juice. Initial weight 3040 g. This animal increased regularly in weight. After about four months, July 29th, 1918, the experiment was terminated as it became evident from this and the previous experiment that 5 cc. of orange juice was an ample dose. The animal weighed 3450 g. at the end of the experiment and was in good health.

Examining the above results it is seen that the daily minimum dose of *fresh* orange juice for the monkey lies between 1 cc.–2 cc. No delay in the onset of scurvy was discerned in the animals receiving 0·5 cc. and 0·75 cc. of the juice, compared with those receiving no antiscorbutic. On the other hand, scurvy was definitely delayed in the animal which received 1 cc. of orange

juice. This is plain both from the time of the onset of the symptoms and from the growth of the animal during the early stages of the experiment. Unfortunately this animal succumbed to some other disease and it was not possible to ascertain the extent to which the severity of the symptoms of scurvy could be modified by the daily administration of 1 cc. of fresh orange juice. The higher doses of 2 cc. or 5 cc. have proved an adequate prophylactic. A monkey therefore of 2–3 kilos is protected from scurvy by about the same quantity of orange juice as a guinea-pig weighing 300–400 g. It is interesting to note that while the minimum dose of antiscorbutic required by the two animals to protect them from scurvy is of the same order, the time taken for the development of the disease is very different, being about two months for a monkey and three weeks for a guinea-pig. This suggests that the monkey possesses a higher store of the antiscorbutic factor than the guinea-pig while their daily requirements for metabolism are equal.

REFERENCES.

Chick and Rhodes (1918). *Lancet*, Dec. 7th, 1918.
Harden and Zilva (1919). *J. Path. Bac.* **12**, 246.
Zilva and Wells (1919). *Proc. Roy. Soc.* B, **90**, 505.

XVIII. THE PRODUCTION IN MONKEYS OF SYMPTOMS CLOSELY RESEMBLING THOSE OF PELLAGRA, BY PROLONGED FEEDING ON A DIET OF LOW PROTEIN CONTENT.

By HARRIETTE CHICK and ELEANOR MARGARET HUME.

From the Department of Experimental Pathology, Lister Institute.

(*Received March 2nd, 1920.*)

(With Plate V.)

The dietary interpretation of the etiology of pellagra has been growing in favour and the disease seems to stand in much the same relationship with the eating of maize as beriberi stands with the eating of rice.

More than 50 years ago, Roussel [1866] pointed out that pellagra can be cured by good food and that without dietetic improvement all remedies fail.

The various theories of its causation which have been advanced are discussed by Funk (1913) who is of opinion that the facts can be best explained by that one which attributes the disease to partial starvation of some important dietary constituent.

Voegtlin [1914], although inclining to the view that pellagra is a chronic intoxication, points out that in the U.S.A. it is endemic only upon a population whose diet is mainly vegetarian and that the possibility of its causation by a vitamine deficiency, or by the defective supply of certain amino-acids in the protein ingested, must be seriously considered.

Lorentz [1914] and Willets [1915] treated advanced cases with mental symptoms by a generous diet with favourable results.

Goldberger [1916] investigated the conditions at two orphanages in Jackson, Missouri, in which cases of pellagra occurred. Some of the children had been admitted pellagrous but others became ill after prolonged residence in the orphanages. Milk and meat were sparingly used in the institutions. By greatly increasing the quantity of meat, milk and milk products, but without disturbing the other conditions, the disease disappeared and failed to recur next spring.

Goldberger, also, made observations upon eleven criminals who volunteered for the following dietetic experiment. The pellagra squad was fed upon a diet consisting of white flour, corn grits, corn meal, corn starch, polished rice, sugar, sweet potatoes, pork-fat, cabbage and turnip-tops. The only animal protein was 4 oz. of meat once a week. The calorific value of the daily diet was 2950 and the prisoners performed only light work. Nevertheless, loss of weight occurred early. After $7\frac{1}{2}$ months cutaneous manifestations were noticed which, in the opinion of experts who were asked to see the cases,

justified a diagnosis of pellagra. These symptoms cleared up on the resumption of a normal diet. Goldberger concludes that the evidence strongly suggests that pellagra will be proved to be a deficiency disease closely related to beriberi but, whatever its nature, it is capable of correction or prevention by including in the diet suitable proportions of fresh animal protein.

Wilson [1916, 1918] had an exceptionally favourable opportunity to study the relation of diet to pellagra in the case of an outbreak of the disease amongst the population of a camp of Armenian refugees at Port Said during 1915, 1916 and 1917. The diet at first supplied was inadequate. It contained only 2200 calories and 92 % of its protein was derived from vegetable sources, of which about $\frac{3}{4}$ was from wheat and $\frac{1}{4}$ from maize. Wilson (using Thomas' [1909] data) calculates that the biological value of the total protein only equalled 22 g. of caseinogen.

Nearly 10 % of the inhabitants of the camp developed pellagra within a year. When, however, the diet was enriched so as to contain 3000 calories and proteins of a total biological value equal to 41 g. of caseinogen, fresh cases soon ceased to occur and the camp remained thereafter free from the disease.

Wilson concludes, from the above observations and from extensive studies of Egyptian and other dietaries, that the cause of pellagra is to be found in an inadequate supply of some one or more of the essential amino-acids and is generally occasioned by relying too exclusively upon cereals, and especially maize, for a supply of protein.

Wilson considers the danger line to be crossed when the total protein intake has a biological value of less than that of 40 g. of caseinogen.

Chittenden and Underhill [1917] produced in dogs a condition which they regard as suggestive of pellagra, by feeding for eight months upon a diet of biscuits, peas and cotton-seed oil. They referred the symptoms to a deficiency of some essential dietary constituent of unknown character. McCollum and his colleagues [1917 to 1919] made a biological analysis of pellagra-producing diets by experiments on rats. They obtained merely evidence of malnutrition and conclude therefore that pellagra is caused by an infection supervening upon lowered vitality due to faulty diet.

A further study of the relation between the incidence of pellagra and dietary was made by Goldberger, Wheeler and Sydenstricker [1918], who have compared the diets partaken of by households in South Carolina villages, in which pellagra occurred, with those of similar households free from the disease. The food consumed by the latter contained more milk, fresh meat, eggs, butter and cheese, and depended less upon cereals for proteins.

The effect of the absence of tryptophan from the protein zein, which forms nearly 50 % of the proteins of the maize endosperm, was demonstrated by Willcock and Hopkins [1907] in the case of young mice, and that of the absence of lysine from the same protein was studied by Osborne and Mendel [1914, 2; 1915]. These two obvious deficiencies, particularly that in trypto-

phan, have frequently been suggested as the cause of pellagra in human beings, the protein of whose diet has been too exclusively derived from maize endosperm. Were such an explanation correct, *i.e.* that pellagra is a lysine or tryptophan deficiency, it would also cover those cases of the disease which are reported in persons who have never touched maize, for doubtless a diet deficient in tryptophan or lysine can be attained by other means than by eating too exclusively of maize endosperm.

The present paper gives, very tentatively, an account of preliminary experiments which were planned in order to make a close study of the symptoms which might develop in monkeys fed on a diet of which the protein was deficient in tryptophan and lysine. The results obtained seemed sufficiently promising to warrant publication.

Experimental.

The diet selected had to satisfy a number of requirements. It had to be adequate from the point of view of fat and of calorific value; it had to contain enough of all the known accessory food factors and it had to be sufficiently palatable for the animals to eat it. It had also to be composed of materials which could be prepared on a large scale, as the experiments were likely to last over many months. The use of purified foodstuffs was thus excluded and the diets had to be so planned as to consist of ordinary foodstuffs or of products of manufacturing processes.

The accessory food factors were supplied throughout as follows:

Fat-soluble *A*. Butter. 10–20 g. per head daily.
Water-soluble *B*. Marmite. 2 g. per head daily.
Anti-scorbutic factor. Orange juice. 10 cc. per head daily.

The fat-soluble *A* ration also provided the fat ration. The orange juice and the marmite were always consumed separately but the butter was mixed with the other food and was not therefore always all consumed. As regards the adequacy of these rations previous experience had shown that 200 cc. daily of milk (about 7 g. of butter fat) would keep a monkey in health over long periods (six months and over) when it was the almost exclusive source of fat-soluble *A* [Barnes and Hume, 1919]. The same amount of fat derived from the same milk ration and about 5 g. of fat derived from wheat germ, of which by no means all was eaten, has been found adequate throughout our scurvy experiments on monkeys.

The antiscorbutic ration was far in excess of that needed to maintain a monkey in health; 2·5 cc. of lemon juice has been found adequate [Chick, Hume and Skelton, 1918], and lemon juice and orange juice, by experiments on guinea-pigs, have been found to have approximately equivalent values (unpublished experinents to which reference is kindly permitted by Miss A. J. Davey).

As regards the water-soluble *B* ration, the amount needed to protect a monkey is quite unknown, but it was judged that 2 g. daily of marmite should

amply suffice, as it evidently did, for the onset and control of any symptoms which occurred were entirely independent of the supply of this factor. The value of the commercial yeast preparation "marmite," for preventing the onset of polyneuritis in pigeons fed on a polished rice diet, had already been demonstrated by us [Chick and Hume, 1917].

The rest of the diet was composed of sugar, cornflour (maize starch, almost protein-free, supplied by the kindness of Messrs Brown and Polson), salt, and corn gluten; a small daily ration of apple or banana was given as a relish. The corn gluten which was almost the sole source of protein in the diet was a product of the manufacture of corn starch; it was similar to that described by Osborne and Mendel [1914, 2, p. 5] for which the following composition is given:

	%
Zein	31·25
Maize glutelin	13·75
Total protein ...	45·00

The remaining 55 % of the corn gluten is oil and carbohydrate.

The product was supplied as a dry cake by the kindness of Messrs W. Mackean of Paisley, and served the purpose well as it was not too unpalatable. No special analysis of it has been attempted, for the figures would have had no value in the interpretation of the results since it has not been attempted within wide limits to determine how much the monkeys consumed.

Table I.

Daily ration offered but not always consumed.

Items common to Diets I, II and III.

Food stuff	Total quantity	Protein	Carbohydrates	Fat
Butter	10–20 g.	0·1–0·2 g.	—	8·4–16·8 g.
Banana	25 g.	0·3 g.	5·5 g.	0·15 g.
or	or	or	or	or
Apple	25 g.	0·1 g.	3·5 g.	0·1 g.
Sugar	26 g.	—	26 g.	—
Orange juice	10 cc.	—	—	—
Cornflour biscuit ...	34 g.	—	34 g.	—
Marmite	2 g.	—	—	—
Totals	—	0·2–0·5 g.	63·5–65·5 g.	8·5–17·0 g.

Table II.

Variable constituents of Diets I, II and III.

	Foodstuff	Total quantity	Protein			Carbohydrate and fat
			Zein	Other protein	Total protein	
Diet I ...	Corn gluten	50 g.	15·6 g.	6·8 g.	22·4 g.	27·5 g.
	Corn starch	30	—	—	—	30
Diet II ...	Corn gluten	32	10·0	4·4	14·4	17·6
	Corn starch	45	—	—	—	45
Diet III ...	Corn gluten	17	5·3	2·3	7·6	—
	Corn starch	62	—	—	—	62

The corn gluten was fed in three different proportions, Diets I, II and III as set out in Table II. Table I shows the food common to Diets I, II and III, Table II shows the starch and protein values (calculated from the above percentage composition for corn gluten) in which Diets I, II and III differ from one another. For the total value of any of the three diets the values in Table I must be added to it and it must be remembered that these figures represent, approximately only, the amount offered to the animals; it is not to be supposed that the total ration offered was consumed for it never was. In experiments such as these the difficulty of getting the animals to consume the monotonous and rather tasteless food for a period of many months is very great; it is a difficulty which it would seem can never be completely overcome, for even if the animal eats the diet well when in health it usually begins to lose appetite when it begins to sicken. The partial starvation which results, with the consequent general lowering of the metabolism, seems to have the effect of moderating the development of the typical and florid symptoms of any deficiency disease. The distaste of a sick animal for the diet also makes it impossible to obtain that rise in weight, following a cure of the symptoms, which is desirable in order satisfactorily to clinch the cure.

Every effort in the present experiments was made to render the food palatable; part of the cornflour was cooked up with the corn gluten into a sort of blancmange and the rest of the cornflour was made into a biscuit through the kindness of Messrs Huntley and Palmer. These efforts were however only moderately successful as partial starvation certainly hastened the decline in weight, particularly in the case of two out of the three monkeys. After symptoms had been alleviated by a modification in the diet it was also found that it was not possible to do more than arrest the fall in weight; the animals did not begin to regain weight until they were put on to a normal diet; on that however they regained weight rapidly, showing that the whole of their trouble was dietary.

The experiments concern three monkeys only, Lazarus, Diana and Jonathan. They all belonged to the species *Macacus rhesus*; this species was chosen because it is one which has an almost hairless, flesh-coloured face, upon which it was hoped that any skin lesion or erythema would show up clearly; much of the rest of the body is dark bluish in colour.

Attempts were made to study the effect of direct sunlight in producing or in increasing any skin lesions that might appear; some results were obtained during the summer but the most important part of the experiment, *i.e.* that in which Diana was upon Diet III, fell in the winter months when sunshine was not available.

Of the three animals, two, Lazarus and Jonathan, lost weight on Diet I (total protein content 23 g.; total protein other than zein 7·3 g.); they were therefore retained on it throughout (Lazarus 92 days, Jonathan 195 days). The third, Diana, liked the corn gluten feed much better and almost maintained weight for 90 days on Diet I and for 26 days on Diet II (total protein

15 g.; total protein other than zein 5 g.). It was not until she received Diet III (total protein 8·2 g.; total protein other than zein 2·9 g.) with its very much lower protein value that she also began seriously to lose weight. The three diets did not differ to any extent in palatability and there seems no doubt that the only reason, at any rate at the commencement of Diet III, for the loss in weight in this case, when at last it did begin, was the lower level of protein intake on Diet III.

The two monkeys, Jonathan and Lazarus, which lost weight on Diet I form one group and the third monkey, Diana, which only began to lose weight when put on to Diet III stands apart. The two first showed only incipient symptoms, the third developed skin lesions of a striking type; the inconclusive skin symptoms of the first two were however identical with the incipient symptoms of the third. It seems probable that Jonathan and Lazarus were suffering from partial starvation, Diana on the other hand was not, at any rate in the earlier stages of the experiment.

A description of the symptoms, together with an account of attempts which were made to perform cures with tryptophan, lysine and caseinogen, will be found described in detail in the protocols; the cures were only partially successful except in the case of Diana, whose cure with caseinogen was of a startling and dramatic nature.

Protocols.

Lazarus. Diet I, Male. Initial weight 2540 g. (see weight curve, Fig. 1). This animal at once began to lose weight on Diet I and on the 51st day showed a faint erythema on the face, particularly on the bridge of the nose, after spending an hour on the roof in direct sunlight (May 15th).

The mouth at this time was rather unclean and the gums inclined to bleed; the animal was subdued and there was some intestinal upset, the abdomen being often distended with gas. It should be noted that this animal was one always rather subject to digestive disorders. The stools were loose, sometimes amounting to diarrhoea.

The erythema, which was of a papillo-macular type, continued intermittently but progressively for the whole of the rest of the experiment. It was never very marked; it left patches which remained faintly pigmented for a few days and sometimes scaled off; it was most marked on the brows, retreating back into the hair, but it was also apparent on the cheeks and on the bridge of the nose and sometimes on the neck, down on to the chest and trunk and even on the inner sides of the thighs.

The appetite was bad and the meals were only partially consumed. About the 85th day the animal was becoming very weak, all the symptoms were aggravated and the mouth was somewhat sore inside and at one corner. The abdomen was very much distended with gas.

On the 89th day the condition seemed very precarious and 0·2 g. tryptophan, *i.e.* the tryptophan from about 13·3 g. of caseinogen, taking the value 1·5 %

tryptophan in caseinogen, as given by Mathews [1916], was given by the mouth.

On the 90th day the animal was much weaker, the lips sore and the diarrhoea bad; 0·2 g. tryptophan was again given and later in the day 3–4 g. of caseinogen with brandy. No improvement took place; the animal was however probably in any case too far gone before a cure was attempted; the next day it was moribund and was chloroformed.

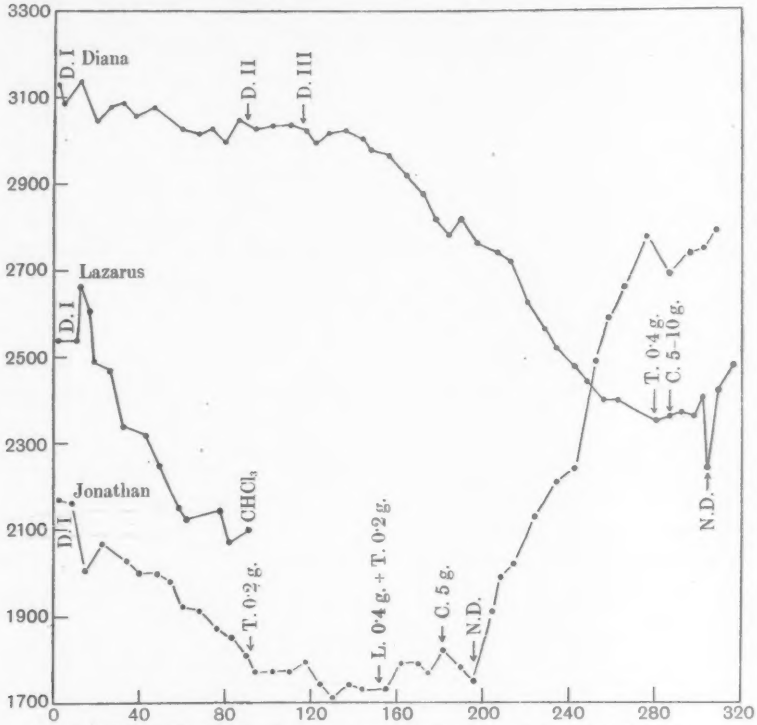


Fig. 1. Weight Chart of the three monkeys, showing dates of changes in the diet.
Ordinates. Weight in grams.
Abscissae. Days from commencement of experiment.
D. (diet), T. (tryptophan), L. (lysine), C. (caseinogen), N.D. (normal diet) indicate day on which the change was commenced.

The post mortem showed nothing remarkable; the body was much emaciated and the caecum and colon greatly distended with gas and soft faeces, but the walls of the intestine showed no special alteration.

There was no sign of tuberculosis or other intercurrent disease.

Jonathan. Diet I, Male. Initial weight 2170 g. (see weight curve, Fig. 1). This animal also began to lose weight from the first. On the 33rd day a dull

reddish patch began to appear about the centre of the lower lip, where the skin and mucous membrane meet, and there were also patches on the scrotum. These were at the time attributed to some skin disease, but they cleared up on a good diet and the patch on the lip closely resembled the patch on the lips developed in the case of Diana.

On the 49th day slight erythema below one eye was noticed but it did not appear to be much enhanced by exposure to sun.

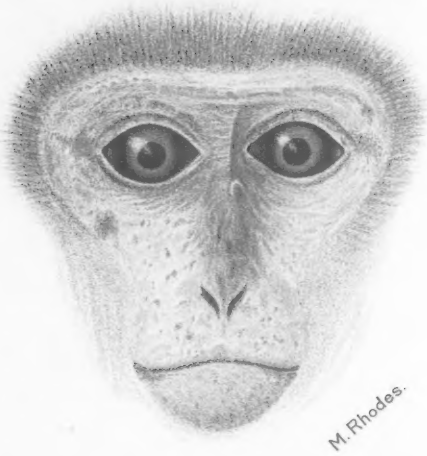
The stools were generally well formed, sometimes rather soft and of a greenish brown colour; diarrhoea was never observed.

The erythema progressed and appeared on the brows and face; on the 71st day it was observed faintly on the chest and on the inside of the thighs. The appetite became bad and the intake of food much diminished, even the ration of banana was often refused. The coat became very scanty, the skin scurfy and chaffy, the gums receded from the teeth but the tongue and inside of the mouth were not sore. The legs were tremulous. The animal had now lost about 20 % of its initial weight and seemed in a very dangerous condition; a daily ration of 0·2 g. of tryptophan was therefore initiated on the 92nd day and continued without any change or addition for 61 days. The loss in weight ceased and the animal seemed brighter and better but no gain in weight took place. Erythema was still recorded from time to time and the animal continued in very poor condition; when the treatment started however there seemed no possible likelihood of life being prolonged for another 61 days if no treatment were given.

On the 153rd day of the experiment 0·4 g. of the unseparated di-amino-acids lysine, arginine and histidine (prepared from caseinogen) was administered by the mouth together with the 0·2 g. of tryptophan already being given; this treatment was persisted with for 29 days up to the 181st day of experiment but no marked change was observed. Addition of further butter and more cornflour failed to make the animal put on weight; probably it liked the diet insufficiently well to eat enough to make it put on weight in any case.

On the 181st day the amino-acids were dropped and 5 g. caseinogen daily were substituted and this treatment was continued for 14 days; still the weight did not rise and on the 190th day it was put on to normal diet of rice, wheat germ, autoclaved milk and cabbage. The weight rose at once by leaps and bounds until it was far above that at the start of the experiment. In 34 days on normal diet the monkey had regained its weight at the start of the experiment and in another 40 days it had increased 500 g. beyond that. Its coat now became thick and glossy; normal change of dentition set in, all trace of skin lesions vanished and the animal, from being depressed and sedentary, became happy and playful. There could be no possible doubt that the whole of its disorder had been dietary.

Although the symptoms in this case were insufficiently marked to be able to say whether a real cure took place or not, it is to be noted that in the



lith. Cambridge University Press

first 92 days of the experiment it lost 20 % of weight and was judged to have reached a precarious condition in which death could not be far off if no change in treatment were instituted. The additions to the diet which are detailed above were then made, starting when the animal's weight was 1750 g.; these additions were continued in all over a period of 103 days, yet no further loss in weight took place after they were instituted and when at the end of the 103 days the animal was put on to normal diet its weight was the same, *i.e.* 1750 g.

Diana. Diets I, II and III. Female. Initial weight 3130 g. (see weight curve, Fig. 1). This animal very nearly maintained weight for 90 days on Diet I, during which period she was occasionally exposed to the sun and very faint erythema of the face was sometimes observed.

On the 91st day the protein and starch were readjusted to the proportions of Diet II which was continued for 26 days; weight was still practically maintained and erythema was not observed but some scurfiness of the skin was present.

On the 117th day Diet III (total protein 8·2 g.; total protein other than zein 2·9 g.) was started and a marked loss of weight set in. Faint erythema with scurfiness of the skin was observed which was intermittent but also progressive.

Menstruation, which had been regular previous to the experiment, ceased and was never observed even on Diet I. The stools were fairly firm and greenish brown in colour.

On the 218th day, the face and brow ridges were distinctly marked with patchy erythema and there was oedema of the eyelids. The spots increased rapidly in brightness, particularly on the bridge of the nose, cheeks, brows and upper lip. A reddish patch of dermatitis began to develop at each angle of the mouth where skin and mucous membrane meet.

Local oedema continued to increase and appeared about the upper lip and face generally. The butter ration was increased and the oedema disappeared for a time, only to reappear later with much greater severity.

All the symptoms continued to progress; the whole skin became very scurfy; the face was often brilliantly red; the mouth showed rough red patches of dermatitis at the angles; these patches however only involved the very edge of the mucous membrane and the actual interior of the mouth appeared normal throughout. The skin of the face evidently itched for the monkey was seen to rub it and scabs were produced in this way. Strong illumination, even from an electric light, brought up the erythema and caused her to hide her face.

On the 253rd day of experiment (137th day of Diet III) the sketch (Plate V) for which we are indebted to Miss M. Rhodes was made.

On the 279th day of experiment (163rd day of Diet III) the animal's condition was deplorable; the oedema which was strictly confined to the area of erythema, i.e. on the face, was so severe that in the morning and evening when

the head was held down in sleep she could scarcely open her eyes; during the day, when the head was held up, the oedema tended to run down under the chin. The animal was very emaciated, having lost nearly 25 % of weight; the face was erythematous all over; the patches of dermatitis at the corners of the mouth were large and very red, the skin was scurfy, the hair almost all gone, the tail was unhealthy looking with patches of dermatitis. On this day 0·2 g. of tryptophan was given by the mouth and by the next day the oedema was distinctly less. The next day 0·4 g. of tryptophan was given and the same dose continued daily for six days; the oedema continued somewhat less but it had always been intermittent; the erythema did not decrease and the animal's condition appeared to be very precarious. Fearing intercurrent disease, 5 g. caseinogen was given which was replaced after one day by the commercial preparation plasmon, this being simply caseinogen in a more palatable form, since much difficulty was experienced in administering the caseinogen.

On the third day from the beginning of the caseinogen treatment, the oedema was almost completely gone, the erythema was reduced, with the nose and eyelids desquamating healthily. The backs of the fingers, parts of the feet and tail also desquamated extensively, showing that some superficial dermatitis must have been present though not easily observable on the dark skin of those parts. Some slight gain in weight took place and the angles of the mouth began to heal; these patches of dermatitis were so severe that, although they began to improve at once, 20 days of the plasmon treatment elapsed before they were fully healed. After 12 days the plasmon had been increased until sometimes as much as 10 g. were taken, but no marked increase in weight ensued. The lesions however completely healed; although the nose remained for long afterwards of a rather purplish red, the oedema had completely disappeared and the whole skin of the face, body, tail, hands and feet was clean. The appetite, however, continued very bad, the animal seemed weak, the weight dropped suddenly and it was decided on the 307th day of experiment (191st day of Diet III), 28 days after the attempts at cure started, to put her on to normal diet, as it was deemed impossible to secure a rise in weight while the same monotonous food was continued. The animal fell upon the food ravenously and a rise in weight at once commenced, strength began to come back and at the time of writing the animal bids fair to show the same return to condition as was shown by Jonathan in similar circumstances [see *Postscriptum*]. Considerable importance is attached to this return to form as it excludes all possibility of tubercular or other infection having played any part in the decline.

Summary and Conclusions.

The foregoing experiments show that three monkeys fed on, or consuming, a low protein diet, whose proteins are at the same time of low biological value, lose weight rather slowly, two possibly from partial starvation through distaste for the diet and only showing mild skin lesions, while the third, which ate well of the diet, at first, showed florid skin lesions and co-extensive localised oedema. The development of these lesions took place after variable, rather lengthy periods.

Attempts to cure with tryptophan failed in one case which was probably too far gone when the cure was attempted, staved off death for many weeks in one case and slightly modified the severity of symptoms in one case.

An attempt to cure with a mixture of lysine, arginine and histidine seemed to have little added beneficial effect in the one case in which it was tried.

Addition of 5–10 g. caseinogen failed in the one very ill case, gave little added benefit in the second case but brought about a dramatic cure in the case where the skin lesions were most severe.

Owing to the small number of animals under consideration, it is not permissible to draw too definite a conclusion; the skin symptoms produced did however closely resemble those of pellagra and there can be no doubt that they were of dietary origin. The skin lesions were bilaterally symmetrical and appeared to be heightened by direct sunlight as in pellagra. They were the only symptom present in all three cases. Diarrhoea and flatulence only occurred in one case out of three, in an animal normally liable to digestive upset. In human pellagra diarrhoea is by no means universal. Nervous symptoms, such as occur in advanced cases of pellagra, were not observed.

As regards the localised oedema which developed in the case of the monkey Diana, which was co-extensive with the dermatitis, this is not uncommon in pellagrins when the dermatitis is acute. The association of general oedema and pellagra has been described by Bigland [1920] among Turkish prisoners of war in Egypt. General oedema has, however, been widespread amongst starving populations during and since the war, but whether it be occasioned by one or many dietetic deficiencies is at present uncertain. A single experiment by Harden and Zilva [1919], in which it followed prolonged feeding of a monkey upon a diet devoid of fat-soluble *A* but otherwise adequate, is an indication.

In the case of our experiments, the diet offered to the animals was deficient only in protein of good biological value but the exact amount of food consumed could not be determined, the quantity uneaten being often considerable, as their appetites became capricious. As to how far the pellagra-like symptoms and wasting were due to an inadequate supply of tryptophan, of lysine or of both, or of some other unappreciated constituent of the protein, the experiments do not supply an answer.

A portion of the expenses of the investigation was defrayed by a grant from the Medical Research Council, to whom our thanks are due.

Postscriptum.

On the 324th day, that is 17 days after the return to normal diet and at a time when the monkey appeared to be rapidly recovering weight and strength, it was found early one morning in convulsions. These recurred at frequent intervals during the day.

Three grains of chloral hydrate were administered and the animal slept, but on waking a further series of epileptic fits succeeded one another at shorter and shorter intervals.

The spasms were entirely bilateral, at first clonic and involving the flexors and terminating with tonic contractions in which the extensors overpowered. Next day the animal was very exhausted, refused food and the intervals between the fits became reduced to a few minutes.

As by evening there seemed little chance of it surviving the night, it was killed by chloroform. At the autopsy there was total absence of fat and the hair had to a large extent disappeared from the body, but the organs all appeared perfectly healthy. There was no abnormality discovered by the naked eye in the nervous system. Portions were taken of various parts for microscopical examination and will be reported upon later by Miss Tozer.

REFERENCES.

Barnes and Hume (1919). *Biochem. J.* **13**, 306.
Bigland (1920). *Lancet*, i, 243.
Chick and Hume (1917). *Proc. Roy. Soc.* B, **90**, 44.
Chick, Hume and Skelton (1918). *Lancet*, i, 1.
Chittenden and Underhill (1917). *Amer. J. Physiol.* **64**, 13.
Goldberger (1916). *J. Amer. Med. Assoc.* **66**, 471.
Goldberger, Wheeler and Sydenstricker (1918). *J. Amer. Med. Assoc.* **71**, 944.
Harden and Zilva (1919). *Lancet*, ii, 780.
Lorentz (1914). *Pub. Health Reports* (U.S.A.), Sept. 11.
McCollum and Simmonds (1917, 1). *J. Biol. Chem.* **32**, 29.
—— (1917, 2). *J. Biol. Chem.* **32**, 181.
—— (1917, 3). *J. Biol. Chem.* **32**, 347.
—— (1918). *J. Biol. Chem.* **33**, 303.
McCollum and Parsons (1918). *J. Biol. Chem.* **33**, 411.
—— (1919). *J. Biol. Chem.* **38**, 113.
Mathews (1916). *Physiological Chemistry.*
Osborne and Mendel (1914, 1). *J. Biol. Chem.* **17**, 325.
—— (1914, 2). *J. Biol. Chem.* **18**, 1.
—— (1915). *J. Biol. Chem.* **20**, 351.
Roussel (1866). *Traité de la pellagra.* Paris, 529.
Thomas, K. (1909). *Arch. Physiol.* 219.
Voegtlin (1914). *J. Amer. Med. Assoc.* **63**, 1094.
Willcock and Hopkins (1907). *J. Physiol.* **35**, 88.
Willets (1915). *South. Med. Journ.* **8**, 1044.
Wilson (1916). Appendix to Report No. 2, On a pellagra epidemic at Armenian refugees camp, Port Said, issued by the Public Health Dept., Egypt.
—— (1918). Report of a committee appointed by the Dir. Med. Serv., Egypt. Exped. Force, regarding the prevalence of pellagra amongst Turkish prisoners of war, Cairo, 20. (Reprinted in *J. Roy. Army Med. Corps*, **33**, 426, 508; **34**, 70.)

XIX. ON THE SWELLING OF GELATIN IN HYDROCHLORIC ACID AND CAUSTIC SODA.

By DOROTHY JORDAN LLOYD.

Report to the Medical Research Committee.

From the Biochemical Laboratory, Cambridge.

(*Received February 19th, 1920.*)

CONTENTS

		PAGE
I.	Introductory	147
II.	Materials and methods	148
III.	The swelling of gelatin in water, hydrochloric acid and caustic soda	148
IV.	The state of gelatin dissolved by acid or alkali in the cold	153
V.	The basic and acidic properties of gelatin	155
VI.	The behaviour of swollen gels in an atmosphere of saturated water vapour	156
VII.	Theory of gel structure deduced from a study of gelatin swelling	162
VIII.	Appendix. On the molecular weight of gelatin	166
IX.	Summary	168
X.	Bibliography	169

I. Introductory.

In 1900 Hardy [1900] put forward evidence to show that gelatin gels are physically heterogeneous, *i.e.* 2-phase systems, and stated in addition that one phase was a solid solution of water in gelatin, the other a liquid solution of gelatin in water. The solid phase (unless the concentration of gelatin were very great) formed an open framework throughout the system.

Procter [1914] discarded the 2-phase theory of gel structure as gratuitous, merely postulating a "molecular network" throughout the jelly. He considered that the equilibrium in a gelatin-acid-water system was due to the chemical combination of gelatin and acid to form dissociated salts, and that the osmotic pressure of these salts determined the volume of the jelly. Procter's theory will be discussed in detail later; at this juncture it may be summarised as a theory which postulates physical homogeneity, but chemical heterogeneity.

In the following pages it is hoped to establish the thesis that both physical and chemical heterogeneity are essential to the gel state, and to show that the volume occupied by a gelatin gel is determined by the resultant of two opposing sets of forces localised respectively in the two phases of the jelly. The experimental evidence will now be considered.

II. Materials and Methods.

Coignet's "Gold Label" gelatin was used for all experiments. An analysis of the sample used showed that it contained 20 % of water, which was removable by drying for 6–8 hours in a hot air oven at 110°. Actually, therefore, there was probably a slightly higher percentage of water, but a correction for 20 % of water only has been made in the initial weights of leaf gelatin taken for the experiments. The gelatin had an ash content of 1·0 %. This consisted mainly of calcium sulphate, with traces of phosphates and chlorides. A small fraction of the base present was combined with the gelatin.

The distilled water used was always of recent manufacture; it was made in an ordinary laboratory still and had a reaction of about $P_H = 5{\cdot}5$. After boiling and cooling the reaction moved to $P_H = 7{\cdot}0$. The acidity therefore was due to dissolved carbon dioxide. Solutions of the gelatin in boiled distilled water gave a reaction $P_H = 5{\cdot}25$, at concentrations of 0·2 and 0·5 %. Since the iso-electric point of gelatin is at $P_H = 4{\cdot}6$, this shows that the gelatin contained a small quantity of base.

In the experiments recorded below discs of leaf gelatin weighing from 0·05 to 0·08 g. were used in most cases. These were placed in Gooch crucibles where they remained at all stages of the experiment. In experiments on equilibria in liquids, both crucible and gel were dried rapidly with filter paper before each weighing. The gels were weighed in their crucibles, but a cover was always placed on the crucible during weighing to check loss of weight by evaporation. The same applies to weighings made during experiments on equilibria in saturated vapour. In drying the gels care was taken to avoid mechanical pressure. The smoothness of the experimental curves appears to justify the simplicity of the method.

The volume of liquid used was in most experiments 150 cc. The experimental liquids were placed in glass bottles with wide necks and ground-in stoppers. The crucibles were easily suspended in these by a harness of fine thread. For experiments conducted in vapour, a glass desiccator was used with distilled water at the bottom. This was partially evacuated, but the air pressure in the chamber does not appear to have an appreciable influence on the shape of the experimental curves.

In all experiments, apparatus in use was kept submerged in a water-bath at 20°. The variation of temperature was $\pm 0{\cdot}25°$.

In all experiments a few drops of toluene were added to the system to prevent the development of bacteria and moulds.

III. The Swelling of Gelatin in Water, Hydrochloric Acid and Caustic Soda.

The swelling of gelatin in water and the influence of acid or alkali in increasing both the rate and degree of such swelling has been described many times by many writers. The rate at which the dry gelatin absorbs water,

and the final amount taken up, depend on a variety of factors among which may be mentioned: hydrogen-ion concentration [Chiari, 1911; Procter, 1914; Loeb, 1918]; temperature [Procter and Burton, 1916]; the ratio of the area of surface of the gelatin to its volume [Spiro, 1910; Fischer, 1910; Hofmeister, 1890; Pauli, 1897]; the ratio of the mass of the gelatin in the system to the mass of dissolved electrolyte (see below), the elastic modulus of gelatin [Procter and Wilson, 1916], and finally, since in every case of absorption of

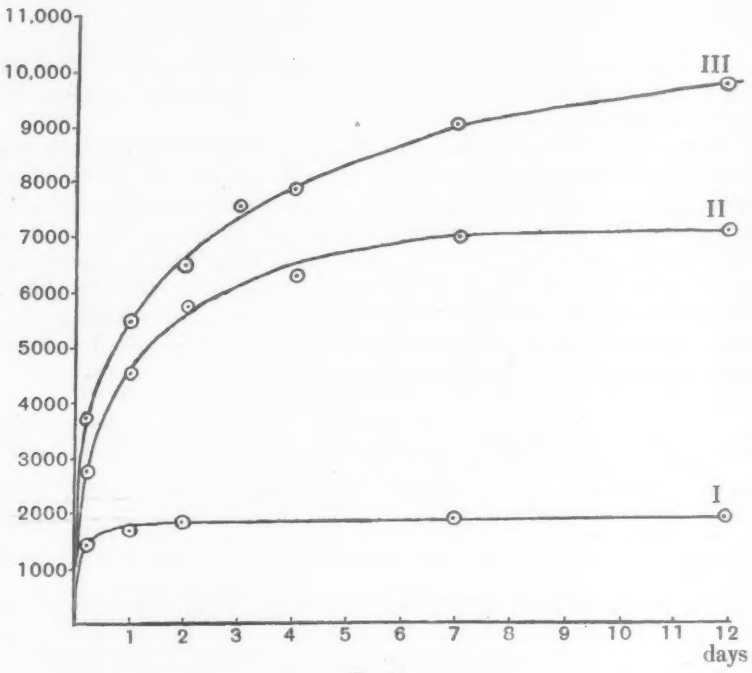


Fig. 1.

Abscissa = time.

Ordinate = weight of gel expressed as percentage of dry weight of gelatin.

water by dry leaf gelatin the initial stage must consist in the restoration of a preformed structure, a factor must be introduced involving the conditions under which the gel was originally formed [see Hardy, 1900]. This last factor is usually unknown. The importance of the mass relations in the system gelatin-hydrochloric-acid-water (other variables being kept constant) is illustrated by the curves shown in Fig. 1. These curves illustrate the progress of swelling in three systems where the ratios $\frac{\text{dry weight of gelatin}}{\text{volume of 0·001}N\text{ HCl}}$ were 0·8/100, 0·8/1000, 0·8/10,000 respectively.

The actual experimental figures for the three systems are:

	I	II	III
Weight of leaf gelatin containing 20 % moisture =	0·820 g.	0·1443 g.	0·0148 g.
Corrected weight of gelatin . . . . . =	0·656 g.	0·1154 g.	0·0118 g.
Volume of N/1000 HCl added . . . . . =	82 cc.	144 cc.	148 cc.
Weight of swollen jelly at equilibrium . . . =	12·04 g.	8·21 g.	—
Weight at equilibrium expressed as percentage of the weight of dry gelatin =	1840	7100	—
Initial reaction of acid, P_H . . . . . =	3·0	3·0	3·0
Final reaction of acid, P_H . . . . . =	4·4	3·57	(3·25)

The intermediate weights of the gel system expressed as percentages of the dry weight of gelatin, and the time relations, are shown in Fig. 1. System III ultimately went into solution.

The influence of the reaction, or hydrogen ion concentration, on both rate and degree of swelling is shown by the curves in Figs. 2 and 3. The problem of the influence of concentration of acid on the degree of swelling has been studied very thoroughly by Procter. It is to be noticed that Procter used 1 g. of moist gelatin in 100 cc. of experimental fluid. In the experiments shown below the ratio of dry gelatin to fluid varies from 1/2200 to 1/3750. The importance of keeping this factor constant unfortunately had not been fully realised at the time the experiment was made. The curves therefore carry a small relative error due to this cause.

	I	II	III	IV	V	VI
Concentration of HCl. Volume in all cases = 150 cc.	0·001N	0·005N	0·01N	0·05N	0·1N	0·5N
Weight of leaf gelatin, g.	0·0640	0·0500	0·0644	0·0852	0·0838	0·0781
Weight of gelatin corrected for water content, g.	0·0512	0·0400	0·0515	0·0682	0·0670	0·0625
Ratio, weight of gelatin/acid volume	1/2930	1/3750	1/2950	1/2200	1/2240	1/2400

The progress of swelling in the six systems can be followed in the curves in Fig. 2. In systems IV, V, and VI the gelatin dissolved in the acid in the course of a few days. System III was unfortunately attacked by mould and the end of the curve was therefore lost, but from other experiments it is known that 0·01N hydrochloric acid also dissolves gelatin in the proportions given. It will be shown later that this is a true case of solution and that it does not involve any break-down of the gelatin molecule.

Curves I and II appear to reach an equilibrium state. The daily increase of weight becomes imperceptible, but even after two or three months the swollen jellies still show a slow, steady increase in weight. This type of behaviour has already been recorded by Masson [1905], studying the absorption of water by silk fibres. It may therefore be characteristic of all proteins. It suggests the possibility that both gelatin and silk fibre would ultimately dissolve in water or dilute acid, given a sufficient quantity of solvent and sufficient length of time.

It is interesting to compare curve I, Fig. 2 with curve II, Fig. 1. The very different level reached in the two cases is probably due to the difference in thickness of the two sheets of gelatin from which the discs were cut in the two different experiments. This factor, *i.e.* thickness of the plate, appears in the equations of both Hofmeister [1890] and Pauli [1897] for the swelling of gelatin in water.

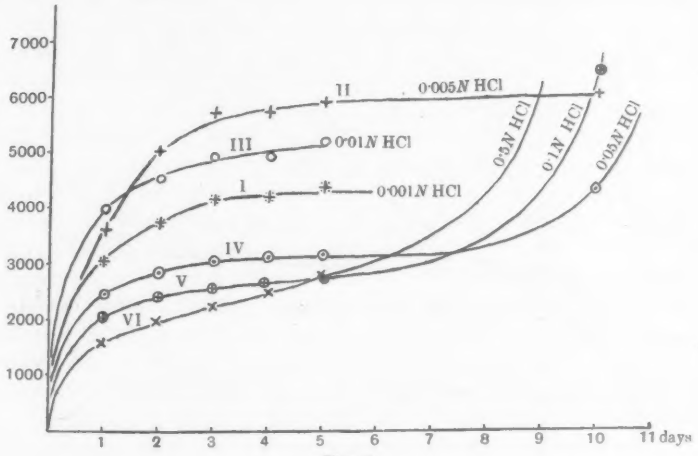


Fig. 2.

Abscissa = time.
Ordinate = weight of gel expressed as percentage of dry weight of gelatin.

In Fig. 3 are given the time-weight curves of the alkaline series. The isoelectric point of gelatin is at $P_H = 4{\cdot}6$. All media having a value for P_H greater than 4·6 are therefore "alkaline" to gelatin. For this reason the curve for distilled water has been placed in the same series as the curves for caustic soda. The experimental quantities used in the experiment were:

	I	II	III	IV	V	VI	VII
Concentration of NaOH. Volume in all cases, 150 cc.	0·5*N*	0·1*N*	0·05*N*	0·01*N*	0·005*N*	0·001*N*	Water
Weight of leaf gelatin, g.	0·070	0·0627	0·0659	0·0595	0·0592	0·0586	0·0801
Weight of gelatin corrected for water content, g.	0·056	0·0502	0·0527	0·0476	0·0474	0·0469	0·0641
Ratio, weight of gelatin/alkali volume	1/2680	1/2980	1/2850	1/3150	1/3160	1/3200	1/2350

The same sheet of gelatin was used for cutting the discs used in all the experiments charted in Figs. 2 and 3. The variation between the two series due to varying thickness of the gelatin has therefore been eliminated. The two series differ in two striking ways; the first is the much greater rapidity with which the gelatin is dissolved in the more strongly alkaline solutions

than in acids of equal normality, the second is the much greater degree of swelling attained by the gels in the acid systems. The last point is well shown in Fig. 4.

In Fig. 4 the curve weight of gelatin gel against reaction of fluid has been plotted. The time has been fixed arbitrarily at 48 hours. The similarity of this curve to the corresponding curve for skeletal muscle [Jordan Lloyd, 1916] is very striking.

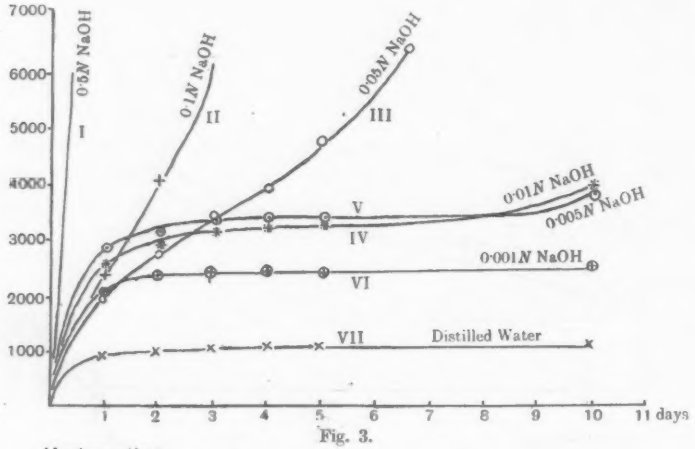


Fig. 3.

Abscissa = time.
Ordinate = weight of gel expressed as percentage of dry weight of gelatin.

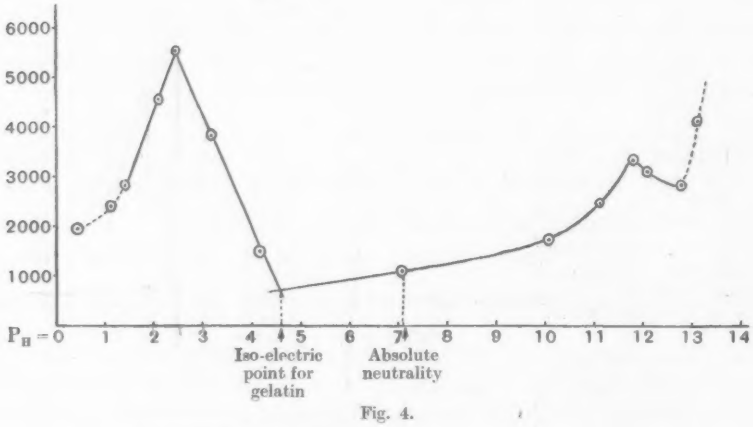


Fig. 4.

Abscissa = hydrogen-ion concentration.
Ordinate = weight of gel expressed as percentage of dry weight of gelatin.

IV. The State of Gelatin dissolved by Acid or Alkali in the cold.

If solutions of gelatin in hydrochloric acid or in caustic soda which have been made by allowing the gelatin to stand in contact with these fluids in the cold are examined *at the moment of solution*, there is no chemical evidence that there has been any break-down of the protein molecule.

Consider first the case of gelatin dissolved in dilute hydrochloric acid. The following experiment may be cited: 2 g. of gelatin were placed in a flask containing 200 cc. $N/10$ hydrochloric acid. The gelatin was completely dissolved after six days at 20°, 20 cc. N NaOH were added to the solution, which was tested and found neutral to litmus, and 220 cc. saturated ammonium sulphate. A white flocculent precipitate was formed. This was filtered off. The filtrate was tested with the biuret test and shown to be free from protein. The precipitate was insoluble in cold water, and was washed several times. It was found to be rapidly soluble in hot water. The precipitate was completely dissolved in 2 cc. of hot water and the solution set to a gel on cooling. A control experiment made by dissolving 2 g. of gelatin in 220 cc. of water with 1·12 g. of sodium chloride behaved in a similar manner.

Gelatin therefore can be recovered from an acid solution.

A similar solution of gelatin (*i.e.* 2 g. in 200 cc. $N/10$ hydrochloric acid) was poured into 3 litres of 96 % alcohol. A white gelatinous precipitate formed which settled slowly to the bottom of the flask. This was filtered off and was washed with absolute alcohol. On cold water being added to the precipitate it swelled rapidly to a clear, glassy, but very soft gel. This was melted and was found to have an acid reaction. The volume was brought to 2 cc. and the solution set to a gel on cooling.

Solutions of gelatin in acid (made in the cold) were compared against control solutions in water for the presence of free ammonia and free amino-groups. Nessler's method was used for the detection of ammonium groups, and Sörensen's formol titration method for the estimation of amino-groups. No difference could be detected between the acid solution and the water solution, *i.e.* there was no evidence of break-down of the gelatin molecule at the moment of solution. If the two solutions are left standing for a day at room temperature, it can be shown that in the acid solution a very slow hydrolysis is taking place.

These facts accord fully with Procter's theory that gelatin forms salts with hydrochloric acid. They also show that the change

$$\text{gelatin} + \text{hydrochloric acid} \rightleftarrows \text{gelatin hydrochloride}$$

is reversible.

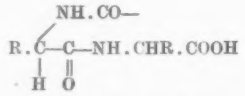
The case of solution in alkali is more complicated. Though the gelatin goes rapidly into solution without molecular break-down, there is evidence that the formation of the solution is accompanied by some internal structural

change in the protein molecule since gelatin is not readily recovered from a caustic soda solution, *i.e.* the change

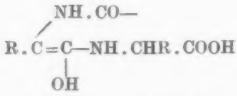
gelatin + caustic soda → sodium gelatinate

is not reversible. Brailsford Robertson [1918] has suggested that acids and bases attach themselves to protein molecules at the —COHN— linkage. He points out that this linkage may exist as an enol-linkage $-\overset{\overset{OH}{|}}{C}=N-$ or as a keto-linkage $-\overset{\overset{O}{\|}}{C}-\overset{\overset{H}{|}}{N}-$, and favours the enol-form since it offers a point of attachment to both acids and bases. A more probable explanation seems to be that under the action of acids gelatin goes to the keto-form, and under the action of bases to the enol-form. This would conform with the observation that the free acid from sodium gelatinate differs in properties from the free base of gelatin hydrochloride. It can also be harmonised with Dakin's theory [1913] that the non-terminal groups in proteins go from the keto-form (1) to the enol-form (2)

(1)



(2)



with loss of optical activity under the action of bases at low temperatures.

The experimental evidence that gelatin dissolves in caustic soda with change of properties, but without molecular break-down, is given below.

0·2 g. gelatin was placed in a flask with 200 cc. *N*/10 NaOH. The gelatin was completely dissolved after two days at 20°. 20 cc. hydrochloric acid were added to the solution, which was tested and found neutral to litmus, and 220 cc. saturated ammonium sulphate. A white flocculent precipitate formed immediately and was filtered off. The filtrate was tested with the biuret test and found free from protein. The precipitate dissolved completely and rapidly in a few cc. of cold water. The solution was reduced to a volume of 2 cc. (= 10 % concentration of gelatin) ... it did not gelatinise on cooling.

A similar solution (*i.e.* 0·2 g. of gelatin in 200 cc. *N*/10 NaOH) was poured into 3 litres of 96 % alcohol. A white gelatinous precipitate was formed which settled slowly. This was filtered off and washed with alcohol. It was strongly alkaline. Enough *N* HCl was added to the gelatinous, slimy mass to make it neutral to litmus. 2 cc. of hot water were added; the precipitate dissolved completely forming a turbid solution but this did not set to a gel on cooling.

Two solutions of gelatin were made:

(A) by dissolving 0·2 g. of gelatin in 200 cc. *N*/10 NaOH in the cold and neutralising with *N* HCl.

(B) by dissolving 0·2 g. of gelatin and 1·12 g. of sodium chloride in 200 cc. water by means of heating.

These two solutions contained identical concentrations of gelatin, sodium ions and chlorine ions, and had the same reaction (neutral to litmus). They were dialysed against distilled water for a week to reduce the concentration of sodium chloride. They were then evaporated to small volume, 2 cc.: B set to a firm jelly on cooling; A remained fluid. The jelly B was melted and the two solutions A and B were half saturated with ammonium sulphate. A dense white precipitate formed in both tubes, and was filtered off. The filtrates were free from protein. Precipitate A was soluble in cold water, precipitate B was insoluble in cold water but dissolved readily on warming.

Gelatin therefore cannot be recovered unchanged from alkaline solution.

Solutions of gelatin in caustic soda, made in the cold, were compared against control solutions in water for the presence of free ammonia and free amino-groups. No difference could be detected between the alkaline solution and the control, *i.e.* there was no evidence of break-down of the protein molecule at the moment of solution. It is important that the solutions should be examined immediately after solution, as the alkaline solution shows evidence of hydrolysis after two or three hours' standing at room temperature. The fresh gelatin-alkaline solutions were examined for their rotatory power. No change in optical activity of the gelatin was detected, and indeed from Dakin's experiments would hardly be expected.

V. The Basic and Acidic Properties of Gelatin.

The observation that gelatin solutions can dissolve considerably greater quantities of lime than can pure water is recorded by Heintz [1853], and the solubility of gelatin in acid or alkali in the cold by Kuhne [1888]. The first definite description of these compounds of gelatin with acids and bases respectively, as gelatin salts, appears to be in a paper by Nasse [1889] published in 1889. Pauli [1912, 1913] further postulated that gelatin salts were ionised in aqueous solution. A considerable amount of work has since been done on the subject and the present position may be summarised briefly as follows: gelatin can combine chemically with both acids and bases, the amount of acid or base combined with unit weight of gelatin being a function of the hydrogen-ion concentration. This is well shown in both Procter [1914] and Loeb's [1919, 1] recent experimental figures. This inter-relation of combined acid and reaction has been interpreted as an adsorption phenomenon, but it can equally well be deduced from the theory that gelatin is a multi-basic acid or a multi-acid base, which dissociates in stages according to the reaction in a way strictly analogous to the dissociation of phosphoric acid. Procter considers that gelatin acts as a di-acid base and has a molecular weight of 839. Consideration of the chemical evidence (see Appendix) shows that the molecular weight of gelatin must be of the order 10,000. A few rough experiments made by means of indicators during the course of this work have indicated that gelatin when acting as a base is not completely neutralised by hydrochloric acid until the reaction is at $P_H < 2{\cdot}5$. At this reaction, and taking

10,000 as the molecular weight, the basicity is 8. Conversely in an alkaline system, the acid valencies of gelatin are not yet satisfied at $P_H = 13$, and the acidity at this reaction appears to be 28. This figure however is not reliable. Guttenberg [1896] and Berrar [1912] using different quantitative methods both reach the figure for gelatin, 10 atoms of nitrogen correspond to 1 molecule of acid. As Berrar states that the binding power of gelatin for acid is unaffected by excess of acid, this value may be taken to be a maximum. Assuming 133 nitrogen atoms in the gelatin molecule (see Appendix) the basicity of gelatin becomes 13.

The chemistry of the gelatin salts is at present under investigation, and it is hoped shortly to publish more satisfactory evidence of their constitution. The theory that gelatin forms ionisable salts which are more soluble in water than either the free acid or the free base is in accordance with all the evidence at present available and has been dealt with briefly here, as it is an essential part of the theory of gel structure which is discussed in Section VII of this paper. The further question of the nature of the ions to which these gelatin salts give rise in solution is also discussed.

VI. The behaviour of Swollen Gels in an Atmosphere of Saturated Water Vapour.

Masson [1905] has recorded for silk fibres that the maximum degree of swelling in liquid water or in saturated water vapour is identical, and that the rates of swelling in the two cases are very nearly so. Schroeder [1903] has stated that this is not the case with gelatin. He gives figures showing that dry gelatin in contact with water vapour will increase its weight up to about 140 % of the dry weight; removed to liquid water further increase up to 1200 % will occur. Conversely gelatin in equilibrium with, or even absorbing water from, liquid water will give up water if transferred to an atmosphere of saturated vapour. Schroeder's experiments were made under apparently controlled conditions, but his controls were not very rigid. Wolf and Buchner [1915] re-investigated the question of the equilibrium of gels in liquid and gaseous water and found that by conducting their experiments in a very reliable thermostat, and using vessels silvered on their internal surfaces, it was possible to transfer gelatin in equilibrium with liquid water into the saturated vapour phase without subsequent loss of weight occurring. With even the slightest temperature variation in the apparatus, loss of water from the swollen jelly promptly occurred. This loss however occurred equally from small basins of pure water suspended in the chamber, and Wolf and Buchner regard it as a distillation on to the walls of the containing vessel under a temperature gradient. It appears therefore to have been satisfactorily demonstrated that the equilibrium of gelatin gels in water and water vapour does not form an exception to the second law of thermodynamics as has been claimed. But in spite of the undoubted truth of Wolf and Buchner's results, and the disproof of Schroeder's phenomenon *per se*, it is hoped to demonstrate

that in systems consisting of acid or alkaline gelatin two separate equilibria do actually occur in the fluid and gaseous media, but that the existence of these two points follows naturally from Donnan's membrane potential theory [1911] and does not therefore form an exception to the second law of thermodynamics.

If gels which are swelling in dilute hydrochloric acid, or in caustic soda, are lifted out of the liquid into a vapour phase, they immediately contract and express fluid. The expulsion of water is frequently so great and so rapid that after three or four minutes in air, drops of liquid can be seen gathering at points all over the surface of the jelly even though the latter be kept in the horizontal plane. In the course of a few hours these beads of water may run together and flow away from the jelly, causing loss of weight, and if the gels are kept in an atmosphere of saturated vapour over a plane surface of water and at a steady temperature, further marked loss will continue to occur for three or four days, doubtless owing to the raised vapour pressure at the curved surface of the fluid drops. It is particularly wished to emphasise that the water does not merely drip away from the lowest edge of a suspended jelly, but is squeezed out at definite *loci* scattered evenly all over the free surface of the gel. This observation confirms the existence of definite pores in a jelly surface, which was postulated both by Bütschli [1896, 1898] and v. Bemmelen [1898]. It may be noticed that Hardy's figures of coagulated colloidal solutions all show an orientation of the solid particles at the surface, which would lead to the formation of pores. This phenomenon of recoil appears to be a good demonstration of the existence of elastic forces within the gel, and it will be shown below that these elastic forces must be localised in the solid framework. The amount of water squeezed out by a gel in this way is independent of the total amount of water in the gel. This is shown by the following experiment: discs of leaf gelatin were soaked in $N/100$ NaOH for 1, 2 and 5 days respectively, the swollen jellies were transferred to the same moist chamber, and weighed every 24 hours.

	I	II	III
Weight of moist gelatin, g. . . . . =	0·0655	0·0590	0·0610
„ „ gelatin corrected for 20 % moisture, g. . =	0·0524	0·0472	0·0488
Volume of $N/100$ NaOH added . . . . =	150 cc.	140 cc.	140 cc.
Days in $N/100$ NaOH . . . . . =	1	2	5
Weight on removing from NaOH, g. . . . =	1·8515	2·1410	2·754
Ditto calculated as percentage of dry weight of gelatin =	3530	4420	5630
Weight after 24 hours in moist chamber calculated as % of dry weight . . . . . =	2520	3590	4440
Do. after 48 hrs . . . . . =	2270	3160	3840
Do. „ 3 days . . . . . =	2180	3000	3550
Do. „ 4 days . . . . . =	1990	2900	—

These figures have been plotted and are shown on the curves in Fig. 5. It will be seen that the three curves are parallel. If the shrunken gels are again immersed in fluid the reaction is reversed, water being again absorbed.

Though the amount of water in a gel does not determine the amount lost in an atmosphere of saturated water vapour, the reaction of the fluid in which the initial swelling of the dry leaf gelatin has taken place is a very potent factor. By varying the reaction it is possible to determine at will whether a gel shall lose weight on being transferred to a vapour phase, gain weight, or remain at a steady equilibrium. Curves for acid gels of varying reaction are shown in Fig. 6, and for alkaline gels in Fig. 7. In these curves, the abscissa is time, and the ordinate the gain or loss in weight which occurs after transferring to the vapour phase. This is expressed in percentages of the dry weight of gelatin. Notice that in all cases the weight at transference is placed at the origin. In both figures a control curve has been plotted. This shows the change in weight of a disc of moist filter paper kept in the same apparatus. Under ideal conditions this should of course run parallel to the x-axis. It will be seen that in reality it falls, slowly and steadily.

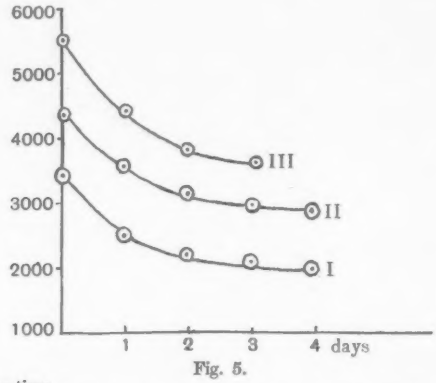


Fig. 5.

Abscissa = time.
Ordinate = weight of gel expressed as percentage of dry weight of gelatin.

The initial weights of the gelatin discs and their weights after soaking for 48 hours in the experimental fluid are given below. The various weight changes in the saturated vapour phase can be read from the curves. 150 cc. of acid or alkali were used in all the experiments. The acid figures (see Fig. 6) are given first.

Concentration of hydrochloric acid, 150 cc. taken	$N/2$	$N/10$	$N/20$	$N/200$	$N/1000$	$N/10{,}000$	$N/100{,}000$
Weight of gelatin disc, g.	0·0676	0·0676	0·0870	0·0695	0·0604	0·0640	0·0650
Do. corrected for 20% water content, g.	0·0541	0·0541	0·0696	0·0558	0·0484	0·0512	0·0520
Weight after 48 hrs. in acid, g.	0·864	1·031	2·0268	2·169	1·430	0·922	0·788
Do. expressed as percentage of dry weight of gelatin	1590	1910	2920	3900	2960	1800	1510

The experimental quantities in the alkaline series (Fig. 7) were:

Concentration of NaOH, 150 cc. taken	*N*/10	*N*/20	*N*/100	*N*/200	*N*/1000	*N*/10,000	*N*/100,000	Dist. water
Weight of gelatin, g.	0·0622	0·0621	0·0620	0·0637	0·0581	0·0636	0·0622	0·0696
Do. corrected for 20% water content, g.	0·0498	0·0497	0·0496	0·0510	0·0465	0·0509	0·0498	0·0557
Weight after 48 hours in NaOH, g.	1·617	1·135	1·206	1·297	0·835	0·501	0·495	0·551
Do. expressed as percentage of dry weight of gelatin	3250	2280	2440	2545	1790	990	996	990

It can be seen from Figs. 6 and 7 how very powerfully the reaction of the fluid influences the behaviour of the gel in the saturated vapour. Though the water loss is considerable in some of the acid systems, it is even exceeded in the alkali systems. In the experiment with *N*/200 HCl (Fig. 6) 100 units

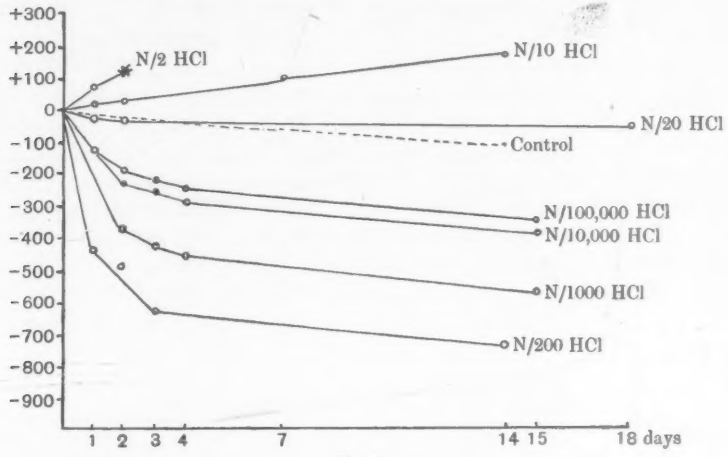


Fig. 6.

Abscissa = time.
Ordinate = change of weight in saturated vapour calculated as a percentage of the dry weight of gelatin.

of gelatin were holding at transference 3900 units of water; 650 of these, equalling one-sixth of the water content of the jelly, were lost in three days. In the alkaline series the greatest loss again occurs at the concentration *N*/200. In the experiment shown in Fig. 7, 100 units of gelatin were holding 3500 units of water; 1700 units of water, equalling nearly a half of the total water content, were expressed in three days. After three or four days the weight curves all run parallel to the filter paper control. Further loss of weight may therefore be attributed to experimental error. It is interesting that the reaction causing maximum swelling in the liquid systems should coincide with the reaction allowing maximum recoil in the vapour phase. It should be noted that the fluid expressed by the gels is not pure water, but contains gelatin

in solution. From acid gels the fluid has an acid reaction; from alkaline gels an alkaline. The reaction of the expressed fluid is not the same as the reaction of the external medium, but lies nearer to the neutral point. This fluid is the "internal phase" of the gel.

Gels which have been swollen in hydrochloric acid or caustic soda of normality $N/20$ or greater all show further absorption of water on being transferred to the saturated vapour. (In the $N/20$ NaOH curve, shown in Fig. 7, there is loss of water followed by absorption.) In some cases the gels

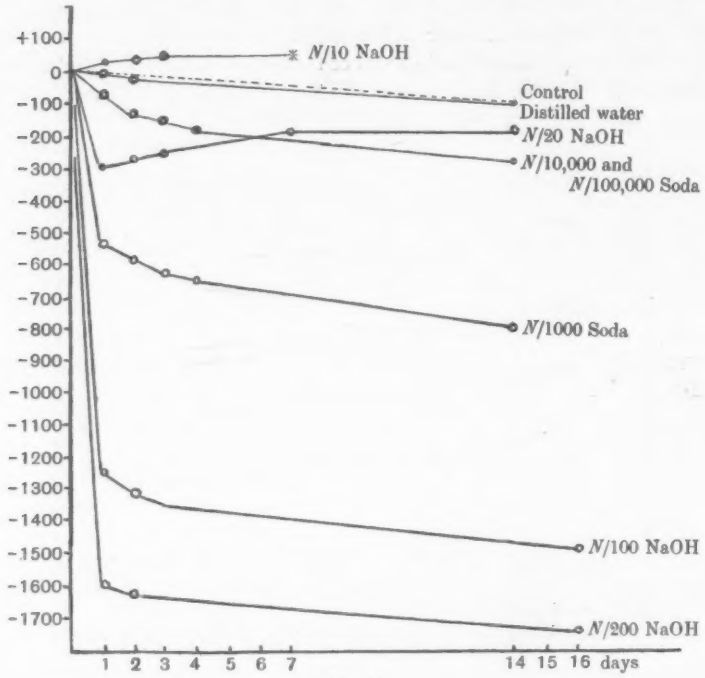


Fig. 7.

Abscissa = time.
Ordinate = change of weight in saturated water vapour calculated as percentage of dry weight of gelatin.

ultimately liquefy. These curves are starred on Figs. 6 and 7. It can be shown here, as in solution in strong acid or caustic soda, that the gelatin has not been hydrolysed but has only gone into solution. It can be recovered from the acid solutions, but not from the alkaline ones.

The behaviour of the swollen gels in a vapour phase seems to be most easily interpreted on the theory that it is determined by a solid framework in the gel, which possesses elastic properties. The stretching force in the gel may be taken to be the osmotic pressure of the gelatin salts in solution. The

forces of recoil appear to conform to Hooke's law, stress $\propto$ strain, *i.e.* they become greater the more swollen the jelly. Osmotic forces and elastic counter-forces will therefore increase in relation to each other, and in the experiments shown in Figs. 6 and 7 it will be seen that with increasing concentration of acid or alkali—up to a reaction corresponding to a concentration $N/200$ for the strong acid hydrochloric, and the strong base caustic soda—that the greater the swelling in the fluid, the greater the recoil in the vapour. Notice that the acid and alkaline systems, though similar, are not symmetrical: the greatest swelling is reached in acid solutions, the greatest recoil in the alkaline ones. As acid and base become more and more concentrated, the gelatin passes to a greater and greater extent from the solid external phase to the fluid internal phase. This gradual corrosion of the solid framework is accompanied by a decrease of elastic properties, until finally they vanish, the framework ceases to exist and the whole system becomes a hydrosol.

The recoil of swollen jellies on transference to a system of saturated water vapour has been quoted as an exception to the second law of thermodynamics. It can however be anticipated from Donnan's membrane theory, which is based on an application of the second law. Donnan has shown that if a system containing in solution an ionisable crystalloid and an ionisable colloid, which dissociates giving one colloidal ion, be separated into two phases by a membrane impermeable to the colloidal ion, then the crystalloid will distribute itself unequally across the membrane, the phase of greater concentration of the crystalloid being the phase free from colloid. Consider the system gelatin jelly in equilibrium with a hydrochloric acid solution. This can be represented as:

$$\begin{array}{cccc|cc} \mathrm{G} & + \mathrm{Cl} & + \mathrm{H} & + \mathrm{Cl} & \mathrm{H} & + \mathrm{Cl} \\ c_1 & c_1 & c_2 & c_2 & c_3 & c_3 \\ & & \mathrm{I} & & \mathrm{II} & \end{array}$$

where c_2 and c_3 represent the concentrations of hydrochloric acid in the jelly and liquid phases respectively, and where c_1 represents the concentration of the gelatin salt. Both salt and acid are assumed to be completely dissociated. Hydrolysis of the gelatin salt will be suppressed in the presence of free acid. If c_1, c_2, c_3 represent ionic concentrations, then c_3 must always be greater than c_2 owing to the electrostatic repulsion between non-diffusible G, the gelatin ion, and diffusible H. But now consider what must happen if the acid solution in phase II be replaced by saturated water vapour. This system is unstable since the electrostatic forces between G and H are no longer balanced. A certain fraction of the hydrogen ions from I, with their accompanying chlorine ions, will therefore be transferred across the membrane, and for this to be possible water in the liquid form must be transferred too. Notice that the greater the ratio c_1/c_2, the greater will be the fraction of c_2 which has to move from I to II in order to adjust the equilibrium, and the greater therefore the loss of water from the swollen gel. Although the weakening of the osmotic forces brought about in this way within the jelly will partially account for

the diminished volume, it is important to notice that the escape of large quantities of liquid water only occurs in gels in which it may be presumed that the elastic framework is still intact. The water is therefore partly expressed under the action of the recoil of the gel.

VII. Theory of Gel Structure deduced from a study of Gelatin Swelling.

Though the gel state is easily recognised in the laboratory, by its appearance and properties, the molecular mechanism which underlies these has been very variously pictured. Three types of mechanical models have been postulated, all differing in their physical conceptions, and all offering evidence in their own support. These are:

(1) The model of a gel as a two-phase solid-liquid system.
(2) The model of a gel as a two-phase liquid-liquid system.
(3) The model of a gel as a one-phase (presumably fluid) system.

In the last model a molecular network is generally thrown through and round the system, in order to cope with the anomaly, that though there is only one phase and that fluid, yet it has lost the property of flowing! The theory that gels in equilibrium with fluids are one-phase structures has been strongly advocated by Procter. It is also the theory finally accepted by Brailsford Robertson after a general survey of the literature of the subject. The one-phase theory of gel structure has been held by Pauli for gelatin, by Hayes [1914] for pecten, etc. The chief line of argument in its favour used by its supporters is that it is sufficient to explain the various properties of gels, and that the appearance of a second phase at setting is an unnecessary assumption.

The two-phase liquid-liquid theory of gels was postulated by Ostwald [1905, 1909]. It is very easy to show that oil and water, for example, can be made, in certain proportions, to form stiff emulsions that will not flow in a test-tube. These emulsions, however, have not really the solid properties of gels. They cannot, for instance, be handled like blocks of gelatin jelly, retaining their shape the while. Hatschek [1916] has shown by mathematical reasoning that the liquid-liquid theory is untenable.

The two-phase solid-liquid theory of gel structure appears in several forms. Hardy, basing his belief on microscopical evidence, states that in most cases gelation consists of the breaking up of the sol into an open solid framework, with an interstitial fluid phase. The surface of the jelly on this theory would appear as a solid membrane perforated by numerous pores. This structure has also been postulated by van Bemmelen and by Andersen [1914] for gels of silica, and is accepted by Freundlich [1909]. In this theory, which is the one accepted by the writer, both solid and liquid phases are continuous.

Another form of the solid-liquid theory of gels is that of Bradford [1918]. Bradford's conception of the gel state is that the solid phase is discontinuous, and he compares a gel in structure to a pile of shot. He states that gels cannot have a rigid framework since they flow slowly and take on the form

of the containing vessel. This observation has not been confirmed during the course of the present work, in fact it has constantly been noticed that thin flat discs of gelatin maintain a rigid attitude when immersed in fluid, and keep perfectly flat for months, even though not lying in a horizontal plane, and unsupported except at the upper and lower edges. Gelatin discs which have been placed in a suspended crucible in order to swell in an experimental fluid will, if they grow sufficiently large, lift themselves right out of the crucible, rather than accommodate themselves to its limitations by bending. Throughout the course of this work, both in liquid and gaseous media, if flowing was observed in a jelly, it was always a sign that liquefaction of the gel was immanent. The theory of the discontinuity of the solid phase of gels appears to be upheld by Arisz [1914].

The process of separation of the solid phase from the liquid has been compared to the process of crystallisation, from the early days of the study of colloids. Both Frankenheim [1835] and von Nägeli [1858] state that the solid part of a gel is crystalline, and the same theory is held by von Weimarn [1908], Levites [1908] and Bradford. Hardy has called the external phase a solid solution, and has shown that it contains water, the amount of the latter depending on the rate of cooling. If the water can be regarded as present in the solid phase as water of crystallisation, and if the existence of several hydrates of gelatin is postulated, then the variation of water content with rate of cooling remains in harmony with the crystalline theory of the solid phase. Moreover, if the external phase is solid then there must be a definite amount of adsorbed water at the solid-liquid interface [see Parks, 1902, 1903]. Any variation in the interfacial area, such as is brought about by variations in the cooling rate, will therefore again affect the water content of the solid phase. Osborne [1918] has recently examined the problem as to whether the solid phase of a gel is crystalline or amorphous. His results are not very conclusive.

Although it is obvious that both the "liquid-liquid" theory and the "pile of shot" theory of gel structure would lead to the formation of highly viscous fluids which would closely simulate gels, yet the theory that gels possess a continuous solid framework appears to explain most satisfactorily the elastic properties dealt with in Section VI of this paper. This continuous framework forms a *locus* for the contracting forces which have been demonstrated to exist in the gels.

There remains to be considered the liquid phase of the gel. Hardy showed this to be a solution of gelatin in water, and Procter brought forward very satisfactory evidence that in a gelatin-hydrochloric acid system the gelatin is present as a soluble salt. Procter's theory of gelatin swelling, though now well known, is summarised here for the sake of clearness. Gelatin is an amphoteric substance which possesses the power of combining with acids to form di-acid, ionisable salts. These dissociate to form one diffusible ion, the anion, and one non-diffusible ion, the gelatin cation. At equilibrium in

a system consisting of gelatin jelly plus acid external fluid, the ionic products on each side of the jelly-water interface are equal, but the sums of the diffusible ions in the two systems are unequal, owing to the unbalanced diffusible anions of the jelly salt which are retained in the jelly phase. This is of course an application to a special case of Donnan's general theory of membrane equilibria [1911]. This inequality of diffusible ions in the two systems causes an osmotic pressure in the jelly, which is greater than the osmotic pressure in the external fluid by a quantity which Procter calls e. This osmotic excess e is the force which causes the jelly to swell, but there must be some other force opposing e, since otherwise the jelly would swell to infinity. The origin of this opposing force is not very clear in Procter's paper, where it is assumed that the jelly only forms a single phase, but if a continuous solid framework is accepted as an integral part of the gel structure, then the counter-force, $-e$, is readily located in the elastic properties of this framework. If e becomes sufficiently great, the framework is stretched beyond its breaking point, $-e$ vanishes, and e does cause the jelly to swell to infinity, in other words to dissolve in the external fluid. The volume occupied by a gel in an acid solution therefore is that due to the resultant of two sets of forces, osmotic and elastic, located respectively in the two phases of the gel. Loeb [1919, 2] has considered it due to the resultant of two sets of forces, osmotic and electric. The obvious importance of the electric forces is included in Donnan's original theory. One common misinterpretation of Donnan's theory might be mentioned here. It has been held that Donnan's "membrane potential" implies that there must be a charge at the surface of the membrane, and various workers, including Ehrenberg [1913], have endeavoured in vain to demonstrate this. An examination of Donnan's theory, however, shows that the membrane potential is due to an electric repulsion between the non-diffusible ions and the diffusible ions *of the same sign*. This leads to a condition of electric strain across the membrane, but not to polarisation in the ordinary sense of the term. Procter claims to have detected a potential of a few millivolts at a gelatin-water interface. The theory of acid-gelatin equilibrium can also be applied to alkaline-gelatin equilibrium, though the qualification must be added that the former equilibrium is reversible, the latter is not. This distinction is probably one of considerable significance in biological problems, especially in view of the fact that the muscle colloids have been shown in freshly excised muscles to be a protein-acid system [Jordan Lloyd, 1916]. It should be noticed before leaving the subject of the gelatin salts that Procter considers that the jelly cation of gelatin hydrochloride is non-diffusible and contributes nothing to the osmotic pressure. But the fact that the internal phase of swollen jellies can be shown to contain gelatin hydrochloride as a non-gelling compound, shows that the colloidal ion must be diffusible and must therefore exert an osmotic pressure. Brailsford Robertson believes that protein salts ionise across the middle of the protein molecule, giving two colloidal ions. If this view were correct, the distribution of free hydrochloric acid across

a jelly water interface would result in equal concentration on both sides. But in Procter's experimental results, the concentration of the acid in the external medium is always greater, at equilibrium, than in the jelly itself, a result which follows naturally if gelatin hydrochloride gives rise to one colloidal and one crystalloïdal ion.

A gelatin gel may therefore be taken to consist of two phases, solid and liquid, and two chemical states of gelatin, *viz.* gelatin *per se* and gelatin in the form of soluble salts. Such gels therefore are three component systems, the components being water, gelatin, and an acid (or base).

It is necessary to consider the evidence that there must be some third component—an electrolyte—in a gel system. The most convincing work on the subject is that of Jordis [1902] on colloidal silicic acid. Jordis shows that the nearer silicic acid comes to chemical purity the more insoluble it becomes, and that ultimately when chemically pure it is practically insoluble in hot or cold water, and cannot be made to pass into the colloidal state. This only occurs in the presence of traces of acid, alkali, or organic groups. Loeb [1918] has shown recently for gelatin that at the iso-electric point it is an inert substance, insoluble in cold water, which moreover releases neither hydrogen nor hydroxyl ions, *i.e.* it has all the characteristics of an insoluble solid precipitate. Consider the case of a hot solution of gelatin cooling at a reaction $P_H = 4{\cdot}6$. The iso-electric gelatin will be precipitated at numerous crystallisation centres, the solid drops will run together to form a framework, but since there can be no osmotic forces in the system (the only available source being the + and − ions of the dilute acid, to which the pores of the framework may safely be presumed to be freely permeable), the framework will contract under the action of its own surface forces, and the internal phase will be squeezed out. In short the system will show minimal swelling. That minimal swelling does coincide with the iso-electric point has been shown by Chiari [1911]. The properties of iso-electric gelatin have not been studied to any extent, probably owing to the extreme difficulty of keeping the system at the iso-electric point, and free from electrolytes. The purest water obtainable is still an electrolyte, and is alkaline to gelatin, and therefore will react with it to form salts. Much work, especially by Dheré [1913], Dheré and Gorgolewski [1910], Nasse [1889], Mörner [1889], etc., has been devoted to preparing ash-free gelatin, and studying its properties. Ash-free gelatin, however, is not necessarily iso-electric, and the fact that Dheré states ash-free gelatin to be electro-negative is sufficient to show that he was dealing with an alkaline system, and not with pure iso-electric gelatin. It is therefore justifiable in view of the work of Loeb, Chiari and others, to deduce that the gel state as a two-phase system cannot exist as a stable system at the iso-electric point.

It is now necessary to consider the fate of the system in strongly acid and strongly alkaline media, respectively. It has been shown during the course of this work that in the presence of sufficient acid or base the hydrogel

becomes a hydrosol. This process is reversible in an acid system, irreversible in an alkaline one. Gelatin, therefore, cannot form gels even in concentrated solutions, if it is present entirely as a gelatin salt. The process of gelation is therefore pictured as follows: gelation will only occur on the cooling of a sol which contains in solution iso-electric gelatin, and gelatin salts in equilibrium with free electrolytes. As the sol is cooled the insoluble iso-electric gelatin is precipitated in a state of suspended crystallisation and forms a solid framework throughout the system. The more soluble gelatin salts remain in solution, and by their osmotic pressure keep the framework extended. Gels therefore are two-phase systems, the solid phase consisting of iso-electric gelatin, the liquid of gelatin in the salt form.

This paper forms a preliminary part of an investigation undertaken on behalf of the Medical Research Committee on the equilibrium of the tissues and body fluids. I have to thank Professor Hopkins for much encouragement during the course of the work.

APPENDIX.

ON THE MOLECULAR WEIGHT OF GELATIN.

The molecular weight of gelatin determined by physical methods has been estimated at values ranging from 800 to 31,000. The following estimations are quoted:

	Mol. wt. of gelatin
Schutzenberger and Bourgeois [1876]	1836
Paal [1892]	900
Procter [1914]	839
Berrar [1912]	823
Biltz, Bugge and Mehler [1916]	5500 to 31,000 for different varieties

Considerations based upon the most accurate estimations of individual amino-acids lead to a figure for the reacting weight of the order of 10,000. The value of the evidence which is given below should be duly weighed. The evidence of the distribution of nitrogen in the gelatin molecule is taken from van Slyke's [1912] estimations.

	% of Total nitrogen
Ammonia nitrogen	2·25
Melanine „	0·07
Cystine „	0·00
Arginine „	14·7
Histidine „	4·48
Lysine „	6·32
Mono-amino „	56·3
Non-amino „ = proline + oxyproline	14·9
	99·02

In order to reduce these percentage figures to others which will show the relations in terms of nitrogen atoms, it is necessary to multiply throughout

by some factor arbitrarily determined. Now the histidine molecule contains three atoms of nitrogen, the arginine four, and the lysine two. The atomic ratios therefore must be such that the histidine nitrogen atoms form some multiple of 3, the arginine of 4, and the lysine of 2. Histidine, being present in very small quantity, may be taken as a convenient basis of calculation. If 1 histidine grouping is present in the gelatin molecule then there must be 3 histidine nitrogens. The percentage histidine value 4·48 can be reduced to 3 by multiplying it by 0·665. If, however, 0·665 is taken as the common factor, both ammonia and arginine yield figures corresponding to fractional (half) molecules. If 2 histidine groupings are assumed, the factor becomes 1·33. Multiplying throughout by 1·33 we obtain:

Ammonia nitrogen	2·9 = 1 × 3	approximately
Arginine „	19·7 = 4 × 5	„
Histidine „	6·0 = 3 × 2	„
Lysine „	8·4 = 2 × 4	„
Mono-amino „	75·2 = 1 × 76	„
Proline + oxyproline nitrogen	20·0 = 1 × 20	„
Total	132·2 = 133	„

These figures are sufficiently satisfactory to justify the assumption that 1 gelatin molecule contains $133x$ nitrogen atoms distributed amongst: $3x$ ammonia (amide) groupings, $5x$ arginine, $2x$ histidine, $4x$ lysine, $76x$ mono-amino, and $20x$ proline + oxyproline groupings. In the absence of further evidence, x may be taken as unity. Two of the figures call for special comment —the lysine value, 8·4 instead of 8·0 is too high, but since the lysine is estimated by difference in van Slyke's calculation, it will carry as a positive error the negative error from both histidine and arginine. The mono-amino figure, 75·2 has been approximated to 76 instead of 75, in order to bring the total from the various groupings to the necessary value of 133.

Nitrogen may be taken to form 18·0 % of the total weight of dry gelatin. The following analyses of purified gelatin are quoted:

Paal [1892]	N = 18·12 %	(ash content = 0·07 %)
Sadikoff [1903]	N = 17·47	Kjeldahl's method
„ [1903]	N = 18·18	Dumas' method
Schutzenberger and Bourgeois [1876] ...	N = 18·3	
Mulder [1843]	N = 18·3	
v. Name [1897]	N = 17·81	
Chittenden and Solley [1891]	N = 18·0	
	Mean = 18·0 %	error = ± 2 %

If 133 atoms of nitrogen form 18·0 % of the weight of the gelatin molecule, then the lowest weight of gelatin which can act as a chemical individual must be $\frac{133 \times 14 \times 100}{18{\cdot}0} = 10{,}344$, or approximately 10,300. The error in the mean of the analyses given above falls within ± 2 %; the error in van Slyke's analyses is of the order ± 1 %; the total error in the computed value is therefore of the order ± 3 %.

SUMMARY.

1. Gelatin is an amphoteric substance which can exist as the free base (or acid), the so-called neutral gelatin, or as a salt in combination with an acid or a base. The molecular weight of gelatin is about 10,000, or some multiple of 10,000.

2. Pure neutral gelatin is only stable at the iso-electric point ($P_H = 4{\cdot}6$); it is slightly soluble in hot water, but insoluble in cold water; but it has the power of combining with water (? as hydrates, or as solid solutions).

3. At $P_H < 4{\cdot}6$ gelatin acts as a base and combines with hydrochloric acid to form soluble, ionisable salts—hydrochlorides. Gelatin is a multi-acid base, and combines with increasing quantities of acid as the reaction of the fluid becomes more and more acid. At about $P_H \overline{=}< 2{\cdot}5$ gelatin exists mainly in the salt form.

The reaction: gelatin + hydrochloric acid $\rightleftarrows$ gelatin hydrochloride is reversible.

4. At $P_H > 4{\cdot}6$ gelatin acts as an acid and combines with soda to form soluble, ionisable salts—so-called gelatinates. Gelatin is a multibasic acid and combines with increasing quantities of base as the reaction of the fluid becomes more alkaline. At $P_H \overline{=}> 13$ gelatin exists mainly in the salt form.

Gelatin acts more readily as an acid than as a base; its dissociation constant for H′ is greater than for OH′, and its valency towards bases is greater than towards acids.

The reaction: gelatin + soda → sodium gelatinate is not reversible.

5. The formation of keto salts in acids and enol salts in alkalies is postulated, ordinary gelatin being taken as a keto compound.

6. The figures given above apply to a system at 20°.

7. Gels are two-phase structures, which can only be formed in the first place from a fluid system (*i.e.* by cooling). They consist (1) of a solid framework of precipitated neutral gelatin, with which is combined a certain amount of water; (2) of an interstitial fluid which is a solution of gelatin in the salt form (either acid or basic).

8. The volume of any system in the gel condition is determined by the equilibrium between two opposing sets of forces. These are:

(1) The elastic forces of the solid framework which tend to make the volume contract.

(2) The osmotic forces of the ionised gelatin salts dissolved in the interstitial fluids which tend to make the volume expand.

9. Neutral gelatin at the iso-electric point cannot make a stable gel, since the constituent of only one phase is present. The gel formed on cooling will be free to contract till it is solid throughout, *i.e.* it will no longer be a gel.

Gelatin in the salt form cannot make a gel, since the constituent of only one phase is present. Gelatin in this form will form only sols with water.

10. The proportion of neutral gelatin to gelatin salts in any system is determined by the reaction of the system.

11. The volume occupied by a gelatin gel swelling in a fluid medium depends on: (1) the ratio of the mass of the dissolved electrolyte to the mass of the gelatin; (2) the geometrical form of the gel; (3) the hydrogen ion concentration of the medium, etc. Gels which are swelling in a weakly acid (or alkaline) medium lose water on being transferred to an atmosphere of saturated vapour. The amount of water lost by the gel is a function of the reaction of the previous external fluid. This water loss is shown to follow from the elastic properties of the gel, and to be in accordance with Donnan's membrane theory.

12. Van Bemmelen's theory that the surface of a gel is to be regarded as a solid membrane, perforated by pores, is confirmed by observation.

BIBLIOGRAPHY.

Andersen (1914). *Zeitsch. physikal. Chem.* **88**, 191.
Arisz (1914). *Kolloidchem. Beihefte*, **7**, 1.
van Bemmelen (1898). *Zeitsch. anorg. Chem.* **18**, 20.
Berrar (1912). *Biochem. Zeitsch.* **47**, 189.
Biltz, Bugge and Mehler (1916). *Zeitsch. physikal. Chem.* **91**, 705.
Bradford (1918). *Biochem. J.* **12**, 382.
Bütschli (1896). *Ueber den Bau quellbarer Körper*, p. 19, Göttingen.
—— (1898). *Untersuchungen über Structuren*, Leipzig.
Chiari (1911). *Biochem. Zeitsch.* **38**, 167.
Chittenden and Solley (1891). *J. Physiol.* **12**, 33.
Dakin (1913). *J. Biol. Chem.* **13**, 357.
Dheré (1913). *J. Physiol. Path. Gén.* **13**, 158, 167.
Dheré and Gorgolewski (1910). *Compt. Rend.* **150**, 934. *J. Physiol. Path. Gén.* **12**, 646.
Donnan (1911). *Zeitsch. Electrochem.* **17**, 572.
Ehrenberg (1913). *Biochem. Zeitsch.* **53**, 356.
Fischer (1910). *Das Oedem*, Dresden.
Frankenheim (1835). *Die Lehre von der Kohäsion*, Breslau.
Freundlich (1909). *Kapillarchemie*, Leipzig.
Guttenberg (1896). *Münch. med. Wochensch.* 147.
Hardy (1900). *Roy. Soc. Proc.* **66**, 95.
Hatschek (1916). *Trans. Farad. Soc.* **12**, 17.
Hayes (1914). *Biochem. J.* **8**, 553.
Heintz (1853). *Lehrbuch der Zoochemie.*
Hofmeister (1890). *Arch. exp. Path. Pharm.* **27**, 395.
Jordan Lloyd (1916). *Proc. Roy. Soc.* **89** B, 277.
Jordis (1902). *Zeitsch. Electrochem.* **8**, 677.
Kuhne (1888). *Lehrbuch der physiologischen Chemie.*

Levites (1908). *Kolloid. Zeitsch.* **2**, 161, 273.
Loeb (1918). *J. Gen. Physiol.* **1**, 41.
—— (1919, 1). *J. Gen. Physiol.* **1**, 379, 483.
—— (1919, 2). *J. Gen. Physiol.* **1**, 717.
Masson (1905). *Proc. Roy. Soc.* **74**, 230
Mörner (1889). *Zeitsch. physiol. Chem.* **28**, 471.
Mulder (1843). *Annalen*, **45**, 63.
v. Nägeli (1858). *Pflanzenphysiologischen Untersuchungen*, Zürich.
van Name (1897). *J. Exp. Med.* **2**, 117.
Nasse (1889). *Jahresber. Thier-Chem.* **19**, 29.
Osborne (1918). *Proc. Roy. Soc. Vic.* **30**, N.S. 153.
Ostwald (1905). *Pflüger's Arch.* **108**, 581.
—— (1909). *Grundriss der Kolloidchemie*, Dresden.
Paal (1892). *Ber.* **25**, 1202.
Parks (1902). *Phil. Mag.* (6), **4**, No. 2, 240.
—— (1903). *Phil. Mag.* (6), **5**. No. 1, 517.
Pauli (1897). *Pflüger's Arch.* **67**, 219.
—— (1912). *Fortschr. naturwiss. Forschung*, **4**, 223.
—— (1913). *Koll. Zeitsch.* **12**, 222.
Procter (1914). *J. Chem. Soc.* **105**, 313.
—— and Burton (1916). *J. Soc. Chem. Ind.* **35**, 404.
—— and Wilson (1916). *J. Chem. Soc.* **109**, 307.
Robertson (1918). *Physical Chemistry of the Proteins*, New York.
Sadikoff (1903). *Zeitsch. physiol. Chem.* **37**, 397.
Schroeder (1903). *Zeitsch. physikal. Chem.* **45**, 109.
Schutzenberger and Bourgeois (1876). *Jahresbericht Thier-Chem.* 30.
Spiro (1910). *Van Bemmelen Festschrift*, 261. Quoted from Mahly's *Jahresbericht*, **40**, 123.
van Slyke (1912). *J. Biol. Chem.* **10**, 15.
v. Weimarn (1908). *Kolloid. Zeitsch.* **2**, 76, 230, 275, 301, 326.
Wolf and Buchner (1915). *Zeitsch. physikal. Chem.* **89**, 271.

XX. THE ANTI-SCORBUTIC PROPERTIES OF CONCENTRATED FRUIT JUICES. PART III.

By ARTHUR HARDEN and ROBERT ROBISON.

From the Biochemical Department, Lister Institute.

(*Received March 2nd, 1920.*)

In a previous paper [1919] a method was described by which orange juice could be reduced, by evaporation at low temperatures, to a crisp, dry solid, retaining in a considerable degree the anti-scorbutic properties of the raw juice. This residue, which was very hygroscopic, was stored in a desiccator over sulphuric acid, at room temperature. Tests on guinea-pigs were carried out when the material was from four to seven months old and showed that a daily ration of 0·5 g., equivalent to about 4·5 cc. of the raw juice, afforded complete protection from scurvy, but the minimum daily amount sufficient to afford such protection was not determined. A quantity of the dried juice was set aside for further tests after a longer interval of time, and these tests have now been carried out. The material formed part of the original quantity prepared in June 1917 and since then had been stored in a desiccator at room temperature. The tests were carried out during April–June 1919 so that the average age of the dried juice was nearly two years. Unfortunately the amount available did not permit of an extended series of tests in order to determine the minimum dose and it was thought advisable to repeat the somewhat high daily ration (0·5 g.) used in the previous tests rather than risk more inconclusive results by feeding single animals on varying smaller amounts. The results of these tests are shown below. In these and other experiments described in this paper the animals employed were guinea-pigs and the basal diet consisted of oats and bran *ad lib.* with 60 cc. daily of milk autoclaved at 120° for one hour.

Table I.

No. of animal	Diet	Initial weight of animal (g.)	Condition of animal during course of experiment	Length of experiment (days)	Final weight of animal (g.)	Result
136	Basal + 0·5 g. dried orange juice daily from the 13th day	326	Weight increased steadily throughout the experiment (maximum weight 519 g. on 75th day). No symptoms of scurvy were observed	77	503	Animal in good health; killed on 77th day; no signs of scurvy
137	Basal + 0·5 g. dried orange juice daily from the 13th day	307	Weight increased steadily throughout the experiment (maximum weight 436 g. on 75th day). No symptoms of scurvy were observed	77	422	Animal in good health; killed on 77th day; no signs of scurvy

It will be seen that the daily ration of dried orange juice was not given until the 13th day of the experiment. This was on account of the limited amount of the material available, but the advisability of the procedure is questionable. The animal's reserve stock of the anti-scorbutic accessory factor must have been very much reduced and this may perhaps account for the somewhat low rate of growth shown by guinea-pig 137.

Microscopical examinations of the ribs of both animals were very kindly carried out by Miss Tozer to whom we are indebted for the following reports:

Guinea-pig 136:—"The two ribs examined are practically normal, a slight shortening of the trabeculae being the only abnormality."

Guinea-pig 137:—"The two ribs examined are normal."

In these experiments, therefore, complete protection from scurvy was afforded by a daily ration of 0·5 g. of dried orange juice, after this had been kept during nearly two years in a dry atmosphere at room temperature. This quantity was equivalent to about 4·5 cc. of the raw juice.

From the work of Chick and Rhodes [1918] and Davey [quoted by Delf, 1920] it would appear that the minimum daily ration of raw orange juice sufficient to protect a guinea-pig completely from scurvy is about 1·5 cc., though it seems probable that this figure may vary to some extent with the character and age of the fruit. Hence the experiments described above do not exclude the possibility of considerable loss of the anti-scorbutic factor either during the process of drying or during storage. It can only be stated that if such loss took place it probably did not exceed 66 % of the amount originally present in the raw juice. It seemed advisable, therefore, to repeat these experiments on a larger scale in order to decide whether any loss of the anti-scorbutic factor actually occurs. Accordingly a considerable quantity of dried orange juice was prepared during November and December 1919. The oranges were fine new season's fruit and yielded a high proportion of juice which was, however, very tart. It was clarified by centrifuging and then passing through filter paper in a Buchner funnel. Five litres of juice containing 10·0 % of total solids were evaporated in quantities of 500–600 cc. at a time, the temperature of the bath being maintained at 50°–52° while the receiver was surrounded by ice. About four hours were required to evaporate this quantity of juice to a thick syrup, the final drying of which was completed by the method previously described. One portion of the dry product has been stored in a screw-top glass bottle, and kept at a uniform temperature of 26°. It is intended to test this portion after a lapse of one year. With another portion animal experiments were at once commenced, two guinea-pigs receiving 0·15 g., two receiving 0·3 g. and two receiving 0·45 g. daily, equivalent to 1·5, 3·0 and 4·5 cc. of raw juice respectively. On account of the tartness of the juice a little cane sugar was added to the ration so that the animals should take it readily. As will be seen from the tables below, all six animals were satisfactorily protected from scurvy during the 85 days that the experiment lasted and we may therefore conclude that no appreciable loss of the anti-

scorbutic factor occurs when orange juice is evaporated to dryness under the given conditions.

Table II.

No. of animal	Diet	Initial weight of animal (g.)	Condition of animal during course of experiment	Length of experiment (days)	Final weight of animal (g.)	Result
156	Basal + 0·15 g. dried orange juice daily from 1st day	283	Weight increased throughout experiment. Maximum weight 550 g. on the 83rd day. No symptoms of scurvy were observed	85	538	Animal in good health; killed on the 85th day; no signs of scurvy
157	Basal + 0·15 g. dried orange juice daily from 1st day	282	Weight increased throughout experiment. Maximum weight 490 g. No symptoms of scurvy were observed	85	490	Animal in good health; killed on the 85th day; no signs of scurvy
158	Basal + 0·3 g. dried orange juice daily from 1st day	283	Weight increased throughout experiment. Maximum weight 573 g. on the 75th day. No symptoms of scurvy were observed	85	555	Animal in good health; killed on the 85th day; no signs of scurvy
159	Basal + 0·3 g. dried orange juice daily from 1st day	332	Weight increased throughout experiment. Maximum weight 627 g. on the 75th day. No symptoms of scurvy were observed	85	613	Animal killed on 85th day. Post mortem examination revealed acute congestion of the left lung but there were no signs of scurvy
160	Basal + 0·45 g. dried orange juice daily from 1st day	283	Weight increased throughout experiment. Maximum weight 557 g. on the 75th day. No symptoms of scurvy were observed	85	525	Animal in good health; killed on the 85th day; no signs of scurvy
161	Basal + 0·45 g. dried orange juice daily from 1st day	300	Weight increased throughout experiment. Maximum weight 506 g. on the 82nd day. No symptoms of scurvy were observed	85	505	Animal in good health; killed on the 85th day; no signs of scurvy

[NOTE:—On the 75th day the animals were moved into another building. As a result of this change five of the animals suffered a temporary fall in weight from which some had not completely recovered when killed on the 85th day.]

That considerable loss of anti-scorbutic potency may occur when other methods of evaporation are employed is shown by the experiments of Givens and McClugage [1919] in which orange juice in shallow dishes was dried by a current of air heated to 55°–60°, the duration of drying varying from 36 to 63 hours. A daily ration of the dried product equivalent to 12·5 cc. of the raw juice was required in order that guinea-pigs might be completely protected from scurvy

whereas on the equivalent of 6·25 cc. the animals developed scurvy in less than 20 days. The loss by this method must therefore have amounted to more than 76 %.

It had been our intention to extend this investigation to the various industrial processes for the rapid evaporation of liquids, particularly those used in the preparation of milk powders, in the hope that one of these processes or some practicable modification might yield from orange juice a product of high anti-scorbutic potency, that could be stored for considerable periods under varying conditions of climate, without serious loss of the accessory factor. Preliminary experiments had been commenced with the co-operation of the Kestner Evaporator Co. Ltd., to whose managing director Mr J. A. Reavell and chemist Mr Nicol we are greatly indebted for much valuable assistance. In July 1919 Givens and McClugage published an account of some successful experiments with dried orange juice prepared by the Merrell-Soule patented process for making milk-powder. The orange juice, mixed with corn-syrup, was forced as a fine spray into a chamber where it met a current of air heated to 75°–80°. The drying was almost instantaneous and the product, when tested on guinea-pigs, was found to afford complete protection from scurvy. The daily dose given to the animals was 1 g. equivalent to 3 cc. of raw juice, which was taken, from the earlier experiments of Chick and Rhodes [1918], as the minimum daily dose of fresh orange juice sufficient to afford complete protection. Givens and McClugage state that they intend to report on the activity of this product after a long period of storage and it is to be hoped that smaller doses will also be tested.

In view of the satisfactory results quoted above it appeared to us unnecessary to continue our attempts, for there would seem to be little doubt that a highly active dried orange juice can be readily prepared on a commercial scale, and from our own experiments recorded in this paper it seems very probable that such a product will retain its potency after prolonged storage under suitable conditions. Orange juice in fact would appear to be particularly suited for the preparation of a dried anti-scorbutic, as it has been shown by Delf [1920] to lose very little of its potency, even when heated at 100° for an hour. The peculiar value of such a substance would lie in its adaptability for infant feeding and for the use of expeditions of long duration, where fresh fruit and vegetables are unobtainable and transport must be reduced to a minimum. The investigation of the effects of storage at tropical temperatures is therefore advisable.

A brief account of the preliminary experiments on large scale evaporation mentioned above may here be given.

(A) The juice of 360 oranges (18·5 litres) was strained through muslin and concentrated in the "Kestner Evaporator," under 25 inches of vacuum and a steam pressure of 10–15 lbs. The time taken for the passage of any portion of the juice through the evaporator was less than one minute and the temperature of the escaping liquid was below 60° (usually below 50°). Owing

to the juice being insufficiently clarified its passage through the inlet valve was somewhat erratic and the consequent evaporation less than the maximum obtainable under the stated conditions of temperature and pressure. It was consequently necessary to pass the juice three times through the evaporator in order to obtain the desired degree of concentration. The product was a viscous syrup containing 68 % of total solids as against 11·6 % in the original juice. A portion of this syrup was tested on guinea-pigs (see Table III, A), and during the course of the experiments was kept in a loosely-corked bottle at room temperature. Two guinea-pigs, each receiving a daily ration of 0·14 g., equivalent to 0·8 cc. of raw juice, developed symptoms of scurvy in 26 days and died in 42 and 39 days respectively. Two guinea-pigs received a daily ration of 0·27 g., equivalent to 1·6 cc. of raw juice. Unfortunately the condition of both animals was unsatisfactory from the first and there was no appreciable gain in weight even during the initial 14 days. In one animal scurvy symptoms were observed after 36 days and the weight commenced to fall slowly. No such symptoms were observed in the other animal though its general condition was not good and its weight declined after the 52nd day. Both animals were killed on the 73rd day and signs of scurvy were found in each case. Two animals received a daily ration of 0·67 g., equivalent to 4·0 cc. of raw juice; one of these was completely protected from scurvy during 93 days but in the other scurvy symptoms were observed after 77 days. From these results it might appear that a loss of anti-scorbutic potency, amounting to more than 63 %, had occurred during concentration of the orange juice. It is very possible, however, that the actual loss due to the process of concentration was considerably less than this, but that further loss occurred during storage in the course of the animal tests, and some evidence in favour of this view can be derived from a comparison of the results obtained with the different doses and with the corresponding amounts of the dried product after further treatment (see below).

(B) The bulk of the concentrated syrup described above was submitted to further concentration in a small vacuum pan fitted with a mechanical stirrer and scraper. A vacuum of 24–25 inches was maintained but the evaporation was very slow and it was found necessary to raise the temperature of the outer bath to 80° for 2½ hours. Even then the result was unsatisfactory, the product being a viscous mass, very difficult to remove from the pan. On cooling it set to a dark coloured, toffee-like solid containing 7·5 % of water.

The final drying of orange juice by this method was obviously impracticable but the product still retained its anti-scorbutic potency in greater degree than might have been expected after such drastic treatment (see Table III, B). Two guinea-pigs, each receiving 0·1 g. daily, equivalent to 0·8 cc. of raw juice, developed symptoms of scurvy in 26 and 31 days and died in 39 and 44 days respectively. A daily ration of 0·2 g. also afforded only partial protection, but two guinea-pigs each receiving 0·5 g. daily, equivalent to 4·0 cc. of

Table III.

A (Concentrated orange juice: total solids = 68 %)							B (Dried orange juice: total solids = 22·5 %)						
No. of animal	Daily ration of "A"	Initial weight of animal (g.)	Condition of animal during course of experiment	Length of experiment (days)	Final weight of animal (g.)	Result	No. of animal	Daily ration of "B"	Initial weight of animal (g.)	Condition of animal during course of experiment	Length of experiment (days)	Final weight of animal (g.)	Result
138	0·14 g.[1] (0·8 cc.)	364	Maximum weight 368 g. on the 12th day. Scurvy symptoms observed on the 26th day	42	217	Death from scurvy on the 42nd day	144	0·1 g. (0·8 cc.)	312	Maximum weight 356 g. on the 12th day. Scurvy symptoms observed on the 26th day	39	207	Death from scurvy on the 39th day
139	0·14 g. (0·8 cc.)	332	Maximum weight 367 g. on the 19th day. Scurvy symptoms observed on the 26th day	39	232	Death from scurvy on the 39th day	145	0·1 g. (0·8 cc.)	312	Maximum weight 323 g. on the 12th day. Scurvy symptoms observed on the 31st day	44	193	Death from scurvy on the 44th day
140	0·27 g. (1·6 cc.)	399	Maximum weight 420 g. on the 19th day. Scurvy symptoms observed on the 36th day	73	301	Animal killed on the 73rd day: severe scurvy	146	0·2 g. (1·6 cc.)	339	Maximum weight 375 g. on the 19th day. Scurvy symptoms observed on the 33rd day	52	233	Death from scurvy on the 52nd day
141	0·27 g. (1·6 cc.)	318	Weight remained constant during 52 days and then fell slowly. No symptoms of scurvy observed but general condition bad	73	241	Animal killed on the 73rd day: signs of mild scurvy	147	0·2 g. (1·6 cc.)	287	Maximum weight 338 g. on the 24th day. Scurvy symptoms observed on the 47th day	73	266	Animal killed on the 73rd day: severe scurvy
142	0·67 g. (4·0 cc.)	349	Maximum weight 442 g. on the 80th day. No symptoms of scurvy observed	93	440	Animal killed on the 93rd day: no signs of scurvy	148	0·5 g. (4·0 cc.)	317	Maximum weight 423 g. on the 50th day. No symptoms of scurvy observed	93	405	Animal killed on the 93rd day: no signs of scurvy
143	0·67 g. (4·0 cc.)	285	Maximum weight 448 g. on the 61st day. Scurvy symptoms observed on the 77th day	93	341	Animal killed on the 93rd day: scurvy not very severe	149	0·5 g. (4·0 cc.)	290	Maximum weight 431 g. on the 78th day. No symptoms of scurvy observed but the animal did not appear in good health. From the 94th day it was given a highly anti-scorbutic diet but its condition did not improve, and it was killed on the 115th day, its weight being 402 g.	93	425	Animal killed on the 115th day. No signs of scurvy were found but there was acute congestion of both lungs

[1] The figures in brackets give the equivalent amount of raw orange juice.

raw juice, did not show any symptoms of scurvy during 93 days, though their general condition was not entirely satisfactory and the normal rate of growth was not maintained throughout the whole period.

SUMMARY.

Orange juice can be reduced by evaporation under suitable conditions to a dry residue without suffering appreciable loss of the anti-scorbutic accessory factor, and this residue still retains a considerable degree of potency after storage during two years in a dry atmosphere at ordinary temperatures.

The preparation on a commercial scale of such a dried orange juice appears to be quite practicable, and should prove of considerable value where an anti-scorbutic material is required in a highly concentrated and stable form.

REFERENCES.

Chick and Rhodes (1918). *Lancet*, ii, 774.
Delf (1920). *Biochem. J.* **14**, 211.
Givens and McClugage (1919). *Amer. J. Diseases of Children* (July), **18**, 30.
Harden and Robison (1919). *J. Roy. Army Med. Corps* (Jan.), 48.

XXI. OBSERVATIONS ON ANTHOCYANINS. I. THE ANTHOCYANINS OF THE YOUNG LEAVES OF THE GRAPE VINE.

By OTTO ROSENHEIM.

From the Physiological Laboratory, King's College, London.

(*Received March 2nd, 1920.*)

These investigations were undertaken with the object of obtaining experimental evidence of a biochemical nature with regard to the formation of anthocyanin pigments by the plant. Until recently two working hypotheses had been advanced in this connection. They both owe their origin to the views of Palladin [1909], who looked upon anthocyanin as a respiratory pigment. Miss Wheldale [1911, 1916] suggested that Palladin's respiratory prochromogens were flavone (or xanthone) derivatives, and expressed their change into anthocyanins in general terms as follows:

Glucosides + water $\rightleftarrows$ chromogen (flavone) + sugar

x chromogen + oxygen $\rightarrow$ anthocyanin.

The first reaction was assumed to be controlled by a glucosidase, and the second by an oxidase. Keeble, Armstrong and Jones [1913] proposed a very similar hypothesis and assumed a preliminary reduction (not necessarily of an enzymatic nature), followed by an oxidation due to the interaction of a peroxidase and an organic peroxide. Both these theories have this in common; they require the presence of oxidases and they assume oxidation as the essential condition for anthocyanin formation. The evidence for the simultaneous occurrence in plants of oxidases and anthocyanins was, however, by no means satisfactory [see Atkins, 1915], and necessitated the further assumption of "inhibitors" of oxidases, if the theory was to be retained. The two theories became finally untenable when it was shown by Combes [1913] and confirmed by Everest [1914], by Willstätter and Mallison [1914], and by Shibata [1919] that the conversion of flavonol derivatives into anthocyanins is not an oxidative process at all, but is due to reduction.

As any hypothesis on the biochemical anthocyanin formation must be based on the chemistry of these substances, a short résumé of our present knowledge of the anthocyanin chemistry is necessary here. A detailed account, based on the work of Willstätter and his co-workers, is given by Perkin and Everest [1918].

It has now been established that the anthocyanin pigments of flowers, fruits and leaves are glucosides and they are the first representatives of a

new class of non-nitrogenous vegetable bases [Willstätter and Everest, 1913; Willstätter, 1914]. They are derived from a reduced β-phenyl-γ-pyrone (*i.e.* 2-phenyl-pyrylium) nucleus (see Formula III). The characteristic of this nucleus is a quadrivalent oxygen atom, which conveys basic properties to its derivatives and enables them to form salts with mineral and organic acids. The methods for the isolation of anthocyanins are based mainly on the formation of these well-crystallised oxonium salts. The differences in constitution of the various anthocyanins are due (1) to the number, nature and position of the carbohydrate radicles attached to the nucleus, (2) to the number and position of phenolic OH groups introduced, whilst (3) methylation of one or more of the OH groups gives rise to further variation. One and the same anthocyanin may occur in three different modifications in the plant, and these modifications may artificially be produced from the isolated crystallised pigment, *viz.* (1) the oxonium salts with mineral or organic acids are red, (2) the phenolic alkali salts are blue and (3) the neutral form, the free oxonium base, is violet. According to Shibata organo-metallic complex compounds of calcium and magnesium are also important factors in the production of flower colour. A fourth colourless modification will be discussed later.

On heating with 20 % hydrochloric acid the anthocyanins are hydrolysed into carbohydrates (glucose, galactose or rhamnose) and their chromogenic components, for which the general name of *anthocyanidins* was introduced by Willstätter and Everest. A very simple reaction makes it possible to decide whether the glucoside, anthocyanin, or the sugar-free pigment, anthocyanidin, occurs in any plant. The reaction depends on the difference which these pigments show in their distribution between amyl alcohol and aqueous acid. The diglucoside anthocyanins are insoluble in amyl alcohol, and are therefore quantitatively retained as oxonium salts in the aqueous layer on shaking their faintly acid solution with amyl alcohol. The sugar-free anthocyanidins on the other hand are soluble in amyl alcohol and are quantitatively removed from their aqueous solution by shaking with amyl alcohol. There are a few exceptions to this general rule, *e.g.* some monoglucosides and rhamnoglucosides are partially soluble in amyl alcohol, but they can be quantitatively removed again by shaking the coloured amyl alcohol solution with fresh aqueous acid.

By means of this reaction it has been established by Willstätter and Everest that all the anthocyanins of flowers, fruits and leaves occur as glucosides, and only in one single instance, the fruit of the grape vine, has it been found that a variable but small percentage of free anthocyanidin is present in the ripe fruit [Willstätter and Zollinger, 1915, 1916].

It was this exception which led me in the first instance, apart from other considerations, to select the grape vine as a suitable subject in which to investigate the relationship of the pigments of the leaves to those of the fruit. The fact that one of the constituents of the pigment exists in the free condition in the fruit in measurable amounts, suggested that such an investigation might throw some light on the mechanism of the synthesis by which

the living plant builds up its anthocyanins. Further, in plants bearing fruits rich in anthocyanin, such as the grape vine, the chances of following the gradual pigment formation during the slow ripening process seemed to be more hopeful than in the case of the flowering plants, in which the pigments are rapidly formed and the pigment carriers themselves are relatively short-lived.

The material for this study was obtained from two established vines, growing in the open, covering several square yards of wall space on my house and producing yearly a large crop of fully matured grapes. On examining the red pigment of the young leaves of these vines, I found the rather remarkable fact that the whole of it was soluble in amyl alcohol and consisted of an anthocyanidin, most probably oenidin, which occurs in combination with glucose as the chief pigment of the purple grape. The pigment itself was obtained in well-formed crystals by a relatively simple method. It was further found that the leaves contained, in an amount equal to that of the preformed red pigment, a colourless modification of the pigment for which the term *leuco-anthocyanin* is proposed. This is convertible into the anthocyanidin by means of hydrochloric acid. The occurrence of anthocyanidin in young leaves appears to be limited to the species *Vitis vinifera*, and presents a Mendelian character of possible value in genetic investigations.

Experimental.

The red pigment of the young leaves of Vitis vinifera. The young red leaves of two varieties ("Black Hamburgh" and "Esperione") were used. They were at first examined separately, but as no qualitative or quantitative difference with regard to their pigments could be detected, the leaves of both varieties were used indiscriminately. The young leaves are produced during the whole of the growing season, and the nature and distribution of their pigments is practically the same in spring and in autumn. Identical results were obtained with the freshly gathered leaves and with air dried material. As the latter has the advantage of smaller bulk and makes its investigation independent of the season, a large quantity of the leaves was collected and dried at ordinary temperature in air. The powdered material was kept for examination in a desiccator over sulphuric acid.

(*a*) *Oenidin.* In some preliminary experiments 5 g. of the fresh leaves (or 1 g. of the dry powder) were ground up finely with 15 g. of clean sand and treated with 40 cc. of 1 % hydrochloric or sulphuric acid. The extract filtered easily through a folded filter and gave a bright wine red filtrate. Of this 10 cc. were shaken gently with an equal volume of amyl alcohol in a separating funnel. A large amount of the pigment present passed into the amyl alcoholic layer and a second extraction removed practically the whole of the pigment. The extracted fluid has a slightly yellowish tint, which appears to be largely due to the presence of flavone derivatives (see later).

The amyl alcohol solution of the pigment is of a fine bluish-red colour and none of the pigment is removed by shaking it with fresh dilute acid (absence of mono- and rhamno-glucosides). On shaking with a dilute solution of sodium acetate the tint becomes more purple, but the pigment remains in the amyl alcoholic layer. On the other hand, dilute sodium carbonate solution removes the whole of the pigment from amyl alcohol, its alkaline solution turning rapidly brown and yellow. On neutralisation with hydrochloric acid in the early stages, the soda solution again turns red and gives up its pigment once more to amyl alcohol. These reactions clearly characterise the pigment as an anthocyanidin.

Preparation. The pigment crystallises remarkably easily, and extracts of leaves made with 1 % hydrochloric acid at a temperature of about 30° gradually deposit the pigment as a microcrystalline sediment on standing in an open flask for some days. The yield of the crude pigment from two extracts is about 2·5 % of the dry material or 0·5 % of the fresh leaves. It is contaminated by a relatively small amount of colourless substances, from which it can easily be freed by repeated recrystallisation from 3 % hydrochloric acid. The pigment is insoluble in this solvent in the cold, but dissolves easily on slight warming. On allowing its solution to cool gradually, the dark red pigment crystallises out in advance of the colourless admixtures. It is filtered under suction, washed with cold 3 % hydrochloric acid and dried in a desiccator over soda lime.

The pigment appears to be pure and consists of uniform crystals when recrystallised several times in the above way. It may be further purified by making use of its solubility in amyl alcohol and by converting it into its well-crystallised picrate. For this purpose the hydrochloride of the pigment is dissolved in slightly warmed 1 % hydrochloric acid and shaken out with amyl alcohol. From its amyl alcoholic solution it is precipitated by adding 2½ volumes of light petrol (B.P. 40°–60°). A bright carmine red watery layer settles out, from which the hydrochloride soon begins to crystallise. The crystals are redissolved by slightly warming the separated watery layer and removing at the same time the dissolved petrol by a current of air. A saturated solution of picric acid in water is added. On cooling, the picrate crystallises in fine deep red prisms, from which the hydrochloride may be easily obtained by the general method described by Willstätter.

This method does not yield the whole of the pigment present in the young leaves, but its simplicity and the relative purity of the product obtained in the first instance make it on the whole preferable to the method subsequently worked out. The latter need therefore only be described in its outlines. It was found that whilst butyl alcohol is an excellent solvent for anthocyanins [see Rosenheim, 1920] it does not dissolve any red pigment from the dry leaf powder in the absence of mineral acid. It removes, however, the whole of the yellow and green pigments (flavones, chlorophyll, etc.). The dry powder is

therefore extracted at ordinary temperature[1] with butyl alcohol until the solvent remains colourless. Extraction is continued with moist butyl alcohol containing 3–5 % hydrochloric acid. The dark red extract is filtered through a layer of sand on a Buchner funnel, and the pigment precipitated with a mixture of ether and light petrol. It is taken up again in amyl alcohol, re-precipitated by light petrol and finally recrystallised several times from warm 3 % hydrochloric acid.

Properties of the crystallised pigment. The hydrochloride of the pigment is obtained on recrystallisation from 3 % hydrochloric acid in well-formed uniform needles, often arranged in rosettes (see Fig. 1). In transmitted light

Fig. 1. Oenidin hydrochloride, the red pigment of young vine leaves.

the crystals, seen singly, are transparent with a brownish red tint; where the crystals are superimposed or cross each other their colour is carmine red. They show a red bronze appearance in reflected light, and are anisotropic under the polarisation microscope. Macroscopically they form a reddish brown non-hygroscopic powder. The purest product so far obtained melts on rapid heating at 200°–202° (uncorr.), on slow heating between 195°–197° (uncorr.). The melting point may possibly be raised on further purification. The crystals dissolve with a brownish red colour in warm water, but the solution becomes rapidly colourless on dilution with water, owing to isomerisation into the pseudo-base. This isomerisation also takes place quickly when the alcoholic solution is diluted with hot water, without the intermediate precipitation of the violet colour base, as in the case of cyanidin. The colour is immediately restored by hydrochloric acid. The salt is readily

[1] Extraction in a Soxhlet apparatus is not to be recommended since the pigment appears to become partially isomerised at the high temperature obtaining during the extraction.

soluble in methyl, ethyl, amyl and butyl alcohol, giving fine violet red solutions. The hydrochloride is easily soluble in 0·1 % hydrochloric acid, very easily if warmed; in cold 1 % hydrochloric acid it is only soluble with difficulty and still more so in 3 % hydrochloric acid, from which it may be recrystallised by allowing its hot solution to cool. The salt is also soluble, especially on warming, in dilute sulphuric acid, even up to 5 % sulphuric acid. With picric acid it forms a characteristic picrate, crystallising in deep carmine red prisms. Ferric chloride gives no colour reaction, and an excess quickly destroys the pigment.

The solutions of the pigment show a single dark band in the green, when examined spectroscopically. The following measurements were taken, when a solution of 20 mg. in 10 cc. butyl alcohol was examined:

5 mm. layer: 530—570; blue absorbed from 420 onwards.
10 mm. layer: 515—585; „ „ „ 430 „

The substance is laevorotatory. The observations were made in white light, and as monochromatic red light was not available no measurements were recorded.

In all its properties and reactions the crystallised pigment isolated from young vine leaves agrees with oenidin, the colour component of the pigment of the purple grape. According to Willstätter and Zollinger, oenidin represents a dimethyl ether of delphinidin, the exact positions of the methyl groups in the molecule being still doubtful. Theoretically, eleven dimethyl ethers are possible, of which only two are known (oenidin and malvidin). It will be necessary therefore to determine not only the number of methoxy groups, but also the products of alkaline decomposition in order to settle the identity of the pigment. Material is being collected for this purpose.

(*b*) *Leuco-anthocyanin.* The presence of a colourless modification of the pigment was first observed during the course of some experiments in which the quantitative distribution of the pigments contained in an acid extract of leaves was investigated. For this purpose 1 g. of the dry leaf powder was extracted with 30 cc. of 1 % hydrochloric acid. 10 cc. of the extract were shaken with an equal volume of amyl alcohol (saturated with 1 % hydrochloric acid) which removed 70 % of the red pigment. Two more extracts with amyl alcohol were made, the third showing only a faint pink tint. The completely extracted residual fluid was compared in a Hellige-Autenrieth colorimeter with the original extract, and the result showed that more than 99 % of the pigment had been removed by amyl alcohol. A more exact estimation of the small fraction left in the solution could not be made, owing to the slightly yellowish tint of the fluid. An attempt was therefore made to determine the minute residual anthocyanin fraction indirectly, by converting it into free anthocyanidin through hydrolysis with hydrochloric acid. On boiling the practically colourless solution with 20 % hydrochloric acid, the surprising fact was noticed that the solution assumed immediately a deep

wine red tint, of apparently the same colour intensity as the original extract. The pigment formed was easily soluble in amyl alcohol with a violet red colour, which could be matched with the original extract in the colorimeter. Its strength was determined and found to be exactly the same as that of the original pigment present.

The experiment was repeated in a modified form with another sample of leaves. In this case the extraction was made with acid alcohol in order to exclude the possibility of the formation of any colourless pseudo-base during the extraction, which was rapidly carried out. From the acid-alcoholic extract the pigment was precipitated by a mixture of ether and light petrol and dissolved subsequently in 0·5 % hydrochloric acid. The solution was divided into two equal parts, of which (1) was hydrolysed with hydrochloric acid and the whole of the oenidin present transferred into amyl alcohol (= total anthocyanidin). From (2) the free oenidin was removed by means of amyl alcohol (saturated with 0·5 % hydrochloric acid) and the colourless residual fluid hydrolysed with 20 % hydrochloric acid (= combined anthocyanidin). This was subsequently extracted with the same volume of amyl alcohol as (1) and the two extracts compared in the colorimeter. The following results were obtained:

(1) Total anthocyanidin = 100.

(2) Combined anthocyanidin = 52.

(3) Free anthocyanidin = 48.

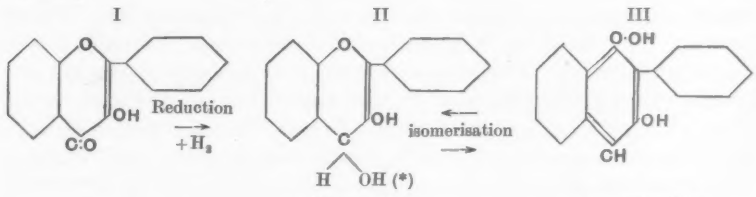
It will be seen that this result agrees well with the previous one and shows that the amount of anthocyanidin present in the colourless modification is about equal to that of free anthocyanidin. This result was confirmed by several other experiments in which various modifications of the procedure were adopted.

The pigment possessed all the characteristics of an anthocyanidin; it remained in the amyl alcoholic layer on shaking with fresh dilute acid, assumed a bluish-violet tint on shaking with dilute sodium acetate solution, and descended quantitatively into dilute sodium carbonate. On rapid acidification of the latter the colour changed into red and the pigment was again given up to amyl alcohol.

On investigating the conditions under which the change from the colourless stage to the pigment takes place, it was found to be independent of the presence of oxygen, for the identical colour intensity is developed with equal rapidity whether the process is carried out in a current of carbonic acid or in air. The chromogenic substance is not extracted by ethyl acetate, after neutralisation of its solution with sodium bicarbonate, thus showing that it does not belong to the rather indefinite group of substances called catechol- or phloba-tannins. These two negative observations are mentioned, since they disprove an assumption, current in the botanical literature of the subject,

according to which the pigments of the grape are formed by oxidation of a colourless oenotannin[1].

In the light of the recent work on the constitution of the anthocyanins, the following suggestion is advanced, as affording an easy and natural explanation of the observed facts. According to Willstätter and his co-workers, the oxonium salts of all anthocyanins and anthocyanidins are hydrolytically dissociated in dilute solutions, giving rise to the formation of the free oxonium base (III) which more or less easily isomerises into the colourless pseudo-base (II). This isomerisation takes place owing to the migration of the basic hydroxyl group from oxygen to carbon. The sugar-free pseudo-bases have been obtained crystallised. They are again transformed into the coloured oxonium salts by acids. The process is therefore a reversible one and according to my observations it is the reverse of this reaction which plays an important rôle in the synthesis of anthocyanins by the plant. It is suggested that the colourless pseudo-bases are the primary products formed by the plant during the reduction of flavonols to anthocyanidins. A consideration of the formulae given below demands, indeed, their formation as a theoretical necessity.



Flavonol — Intermediate Colourless Pseudo-base — Anthocyanidin (colour base)

This reduction has been successfully carried out in the laboratory by Willstätter and Mallison [1914] and, since I have now shown that the free anthocyanidin occurs in young vine leaves, it is not surprising to find also the intermediary product. In the young leaf, however, the pseudo-base does not occur in the free state, but in combination with either a carbohydrate or possibly another complex. For this combination the general name *leuco-anthocyanin* is proposed. If we assume that the carbohydrate (or similar complex) is attached to the pseudo-base by means of the hydroxyl group marked * in II, one can easily understand that the change from the colourless modification into the basic pigment cannot take place until the carbohydrate radicle is split off. This hydrolysis is rapidly effected by strong acids, but only very slowly if at all by 1 % hydrochloric acid. In the presence of the strong

[1] On the other hand, however, I have obtained certain experimental evidence which points to a relationship of a different character between anthocyanins and phlobatannins and which suggests the existence of reduced flavonol rings in the latter substances. Additional support for this suggestion is afforded by the correlation in the distribution of tannins, flavonols and anthocyanins, and by the fact that they all three furnish identical cleavage products on alkaline decomposition (phloroglucinol and various hydroxy-phenolic acids).

acid, the liberated pseudo-base immediately isomerises into anthocyanidin. An attempt to effect hydrolysis by means of emulsin has so far not yielded any decisive results. The chromogenic substance is not extracted by ether even after saturation of its solution with ammonium sulphate. As the free pseudo-bases are soluble in ether, under these conditions, their absence is proved, and this fact also shows incidentally that the colourless modification is not artificially produced, during the preparation of the extract, by the isomerisation of preformed anthocyanidin.

I have so far been unable to isolate the leuco-anthocyanin itself from the young leaves of the grape vine. I found, however, that the unripe berries of the purple grape and the ripe berries of white grapes contain leuco-anthocyanin in much larger amounts and from this source it may be easily obtained, so far only in an amorphous condition. (Further details will be given in a future communication.)

It is interesting to note that Willstätter and Nolan [1915] recorded an unexplained observation in their work on the pigment of *Rosa gallica*, which points to the existence of leuco-anthocyanins in this flower. They noticed that an acid methyl alcoholic extract of the flower showed, on standing, the remarkable phenomenon of gradually increasing its colour to twice its original intensity. They were unable to explain this phenomenon as being due to the occurrence of the pseudo-base of cyanin, since this rapidly changes into the coloured salt under these conditions. It was also not due to oxidation since it occurred equally in the absence of oxygen. They assume the presence of an unknown anthocyanin, the colourless isomer of which is slowly changed into the coloured salt.

Oenidin as a Mendelian Factor. The occurrence of free anthocyanidin as the only red pigment of young leaves in *Vitis vinifera* naturally suggested an investigation of the pigments of young leaves of other plants, in order to find out whether this occurrence represents a general stage in the formation of anthocyanins by the plant organism. For this purpose I examined the pigmented leaves of 85 different species of various families, the enumeration of which would occupy too much space. For a large selection of suitable material I am indebted to the authorities of the Royal Botanic Gardens at Kew, and especially to Mr Dallimore, who also kindly supplied the botanical names. In most cases the young as well as the autumnal leaves were examined. The fresh material was ground with clean sand and extracted with 1 % sulphuric acid. A clear filtrate was obtained in most cases, 5 cc. of which was shaken out with amyl alcohol. The result showed that in all the varieties of *Vitis vinifera* examined, the pigment of the young leaves was soluble in amyl alcohol, whilst all the other plants contained only anthocyanins, insoluble in amyl alcohol, in both their young and autumnal leaves. In a few cases (several species of *Euonymus* and *Geranium*) the amyl alcohol extract was slightly pigmented. The pigment, however, was easily removed by fresh dilute acid, thus proving the absence of anthocyanidins.

It would appear therefore that the occurrence of free anthocyanidins is limited to *Vitis vinifera*, and it became of interest to examine other species of the *Vitis* family. As is well known, *Vitis vinifera* is the only European species of this family, whilst at least 18 species are known in America and about eight in Asia. I was able to examine six Asiatic species (*V. pulchra, Thomsonii, Wilsonii, amurensis, Baileyana* and *Coignetiae*) and three American species (*V. arborea, aestivalis* and *Bourquiniana*). They all gave negative results with the single exception of *Vitis Bourquiniana*. In this case the pigment of the young leaves was to a large extent soluble in amyl alcohol and consisted of anthocyanidin.

This result is rather suggestive in so far as the origin of *V. Bourquiniana* and its recognition as a species has given rise to a great deal of discussion in viticultural journals[1]. According to the standard work on American grapes by Hedrick [1907], the name *V. aestivalis Bourquiniana* was given by Munson, who ranks a group of several similar varieties as a species, in honour of the Bourquin family of Savannah, whose ancestors were supposed to have brought them from France to America over 150 years ago. Munson's derivation of the origin of this vine has not been accepted by either French or American botanists, and the general opinion appears to be that *V. Bourquiniana* is a hybrid between *V. aestivalis* and some form of *V. vinifera*.

This conclusion seems to receive an experimental confirmation by the above described reaction, which clearly demonstrates the influence of *V. vinifera* on the nature of the pigment of the supposed hybrid. If further investigation of other species of *Vitis*, which I hope to carry out, should confirm the observation that the occurrence of free anthocyanidin is limited to *Vitis vinifera*, this biochemical test might prove useful for investigations of genetic problems.

Summary.

(1) The red pigment of the young leaves of the grape vine has been isolated in a crystalline form. It is most probably identical with oenidin, the non-glucosidic component of the pigment of the purple grape. This is the first known, and so far the only instance, in which the red pigment of leaves consists of free anthocyanidin.

(2) The occurrence of a colourless modification of the pigment has been demonstrated, for which the general name *leuco-anthocyanin* is proposed. It is present in combination possibly with a carbohydrate or other complex, and is converted into anthocyanidin by strong acids.

(3) The European species *Vitis vinifera* appears to be the only representative of the family *Vitis*, which is characterised by the production of free anthocyanidin, and the bearing of this observation on genetic problems is discussed.

[1] I am greatly indebted to Mr E. A. Bunyard, of Maidstone, for kindly referring me to the extensive literature of this subject.

REFERENCES.

Atkins (1915). *Proc. Roy. Soc. Dublin*, **14**, 317.
Combes (1913). *Compt. rend.* **157**, 1002, 1454.
Everest (1914). *Proc. Roy. Soc.* **87** B, 444.
Hedrick (1907). *The Grapes of New York, Rep. N.Y. Agric. Stat.*
Keeble, Armstrong and Jones (1913). *Proc. Roy. Soc.* **86** B, 308.
Palladin (1909). *Biochem. Zeitsch.* **18**, 151.
Perkin and Everest (1918). *The Natural Organic Colouring Matters* (London).
Rosenheim (1920). *Biochem. J.* **14**, 73.
Shibata, K., Shibata, Y., and Kasiwagi (1919). *J. Amer. Chem. Soc.* **41**, 208.
Wheldale (1911). *J. Genetics*, **1**, 133.
—— (1916). *The Anthocyanin Pigments of Plants* (Cambridge).
Willstätter (1914). *Ber.* **47**, 2831.
—— and Everest (1913). *Annalen*, **401**, 189.
—— and Mallison (1914). *Sitz. Ber. Preuss. Ak. Wiss.* **29**, 769.
—— and Nolan (1915). *Annalen*, **408**, 1.
—— and Zollinger (1915). *Annalen*, **408**, 83.
—— —— (1916). *Annalen*, **412**, 195.

XXII. THE ACTION OF THROMBIN UPON FIBRINOGEN.

By JOHN OGLETHORPE WAKELIN BARRATT,

Beit Memorial Research Fellow.

From the Lister Institute.

(*Received February 27th, 1920.*)

Method.

The solutions of fibrinogen, thrombin and sodium chloride employed in these experiments contained about 0·5 % of tri-sodium citrate.

As source of fibrinogen human plasma was ordinarily used, being prepared by mixing 12 cc. of blood, immediately after withdrawal from a vein of the forearm, with 1 cc. of 0·85 % sodium chloride solution to which 0·13 g. sodium citrate had been added. After centrifugalisation the plasma was pipetted off and stored at 0°. In a few experiments fibrinogen was precipitated by pouring human plasma into twenty volumes of distilled water through which a stream of carbon dioxide had been passed, the precipitate being separated by centrifugalisation and then redissolved in citrated 0·85 % sodium chloride solution.

As source of thrombin freshly prepared whipped blood, to which 1 % of sodium citrate had been added as soon as separation of fibrin was completed, was used in a few experiments. As, however, the range of concentration of thrombin obtainable in this way was small, in most experiments the thrombin employed consisted of the venom of *Echis carinatus*[1] dissolved in 0·85 % sodium chloride solution, to which 0·5 % of sodium citrate had been added [Barratt, 1915]; the limit of utility of this form of thrombin was about 1 in 30,000,000 and the highest concentration used rarely exceeded 1 in 3000; it was completely destroyed by heating to 75° for ten minutes.

Dark background illumination was obtained by means of a paraboloid condenser with central stop, an arc lamp being used as source of light.

Coagulation was usually studied at room temperature, the mixture of fibrinogen and thrombin, which formed the subject of observation, being placed upon a glass slide, within an area ringed with vaseline, and closed by a coverslip: the thickness of the layer of fluid examined was usually about 50μ. Occasionally coagulation was carried out in small glass tubes.

[1] For a supply of this I am indebted to the kindness of Dr C. J. Martin, Director of the Lister Institute.

Fibril formation.

When a liquid containing fibrinogen is mixed with a solution of thrombin in the presence of sodium citrate or oxalate coagulation occurs, due to the formation of fibrin fibrils. If the production of such fibrils occurs with sufficient slowness the course of the process can readily be studied[1].

Fig. 1 represents the appearance, under a dark background illumination, of a mixture of (1) citrated human blood (containing fibrinogen, but no thrombin), and (2) whipped human blood[2] (containing thrombin, but no fibrinogen): at the end of three hours a fine fibril of fibrin, about 0·17μ thick, previously invisible or on the threshold of visibility, was observed

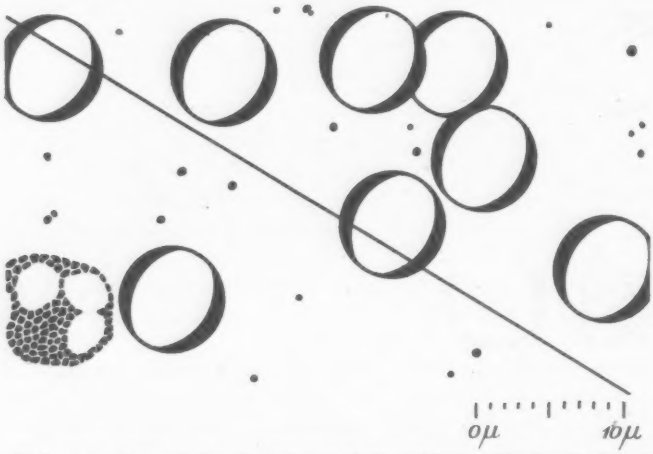


Fig. 1. Early stage of coagulation of a mixture of citrated human blood and whipped human blood (subsequently citrated). Film preparation three hours old. A single fibril is seen. In addition to red cells highly refractile dancing particles and a polymorphonuclear leucocyte are seen. ×2300.

lying across the field. At the end of a further period of twenty hours, this fibril was found to have increased considerably in thickness, while a second delicate fibril, about 0·2μ thick and 25μ long, had appeared (Fig. 2): the length of the large fibril was found on measurement to be 0·342 mm. Fibrils vary in length from 20μ or less to 500μ or more; their apparent diameter ranges from about 0·17μ to 1·1μ; the smallest fibrils do not present any

[1] Fibril formation has been investigated ultramicroscopically by several observers, two views having been advanced as to the nature of the process occurring. According to Mayer [1907] and Cesana [1908] coagulation consists in the appearance of very fine granules, which increasing in size arrange themselves in rows, forming threads, subsequently becoming numerous and thus giving rise to a network. Stübel [1914] compares coagulation to a crystallisation process and Howell [1914, 1916] states that fibrin is deposited not as a network, but as separate needles or crystals, which are massed to form a close meshwork.

[2] Citrated after separation of fibrin.

obvious structure, but the largest exhibit an irregular imperfectly reticular aspect (Fig. 3 *c*), due to refractile material, which is in more or less marked contrast to the rest of the substance of the fibril, the pattern thus produced sometimes extending along the whole of the fibril, sometimes being observed in short lengths of fibril, the intervening portion being apparently homogeneous. No further increase in size or number of fibrils could be recognised subsequently in the microscopic field shown in Fig. 2.

Two processes are therefore concerned in the formation of fibrin fibrils: (1) production of primitive fibrils; and (2) subsequent growth of these in presence of fibrinogen. The number of the finest fibrils actually seen depends

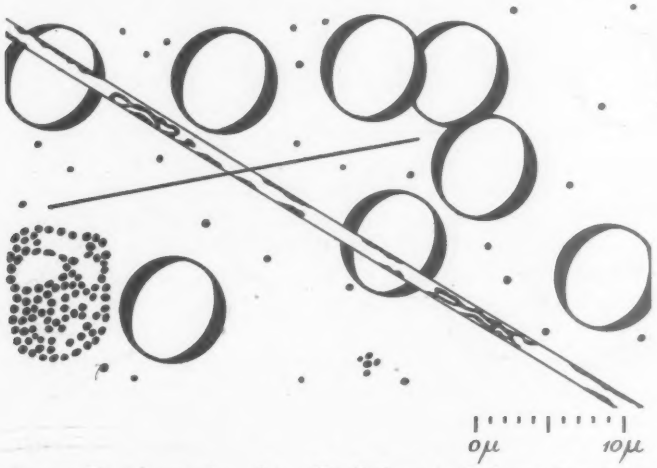


Fig. 2. Same as Fig. 1, twenty hours later. The fibril seen in the former figure has increased considerably in size and a second fine fibril has appeared. The leucocyte has become swollen and its granules, which were fixed in the preceding figure, are now in oscillatory motion. ×2300.

upon the intensity of the illumination employed: with a poor arc light only a few fine fibrils are seen; the more intense the arc, the larger the number of exceedingly fine fibrils which can be rendered visible. The possibility must therefore be borne in mind that in mixtures of fibrinogen and thrombin excessively small fibrils may be already in existence, which cannot be rendered visible until their thickness has been increased by further addition of fibrin. It will be shown later that the exact moment at which fibrils are first formed can be studied only by indirect methods (p. 201). On the other hand the time of first appearance of visible fibrils can be determined by direct observation: to this method of investigation most of the results obtained in the present work are due.

In order to determine the relative amounts of fibrinogen and thrombin taking part in coagulation the following experiment was made.

Exp. A. Equal volumes of citrated human blood plasma (fibrinogen solution) and citrated 1 in 8,000,000 venom (thrombin) solution were mixed together. Coagulation occurred at room temperature (18°). At the end of sixteen hours a portion of serum expressed from the clot was mixed with an equal volume of venom solution (1 in 100,000) and allowed to evaporate at room temperature to about one-tenth of its bulk: no further coagulation occurred.

The amount of fibrinogen contained in the plasma employed was determined by precipitation with twenty volumes of distilled water through which a stream of carbon dioxide had been passed, the precipitate being then dried to constant weight at 120°. The plasma contained 0·78 % of fibrinogen[1].

In the above experiment the weight of fibrinogen employed was more than 60,000 times as great as that of the venom added. It follows therefore that fibrin arises by transformation of fibrinogen: thrombin merely acts as a determinant, that is to say catalytically[2].

Fibrils of fibrin are frequently attached to each other and may coalesce over part of their length, but no branching occurs (*cp.* Figs. 1 to 3). When present in sufficiently scanty numbers they are observed to be quite separate and to be unconnected with any of the suspended particles present, so that their characters can be readily studied, especially if the conditions of experiment are such as to lead to the production of fibrils of relatively large size: they are then seen to be attached to the surfaces of glass bounding the layer of liquid in which they have been formed.

Whatever may be the ultimate mode of production of these fibrils it is obvious that they form separate entities and that they are therefore particulate in origin, their appearance being determined by the addition of thrombin and the material of which they are constructed being provided by fibrinogen. Such particles of origin appear to be amicroscopic, for none of the particles seen in plasma or solution of fibrinogen can be observed to disappear or diminish in number after fibril formation is completed, and again the number of particles of origin, estimated from the number of fibrils developing, is very considerably in excess of the number of particles ordinarily observable in fibrinogen solution by ultramicroscopic methods. Presumably these particles of origin of the fibrils may be contained either (1) in the venom solution or (2) in the fibrinogen solution, employed in experiments on fibril formation. As a preliminary step in the investigation of this point the appearance presented at the junction of a solution of fibrinogen with a liquid containing thrombin was next studied.

[1] *Cp.* note, p. 197.

[2] Howell [1910] concludes that thrombin probably does not act upon fibrinogen after the manner of an enzyme. The solution of thrombin employed by this observer could, however, be heated to the boiling-point without completely losing its coagulative action on fibrinogen.

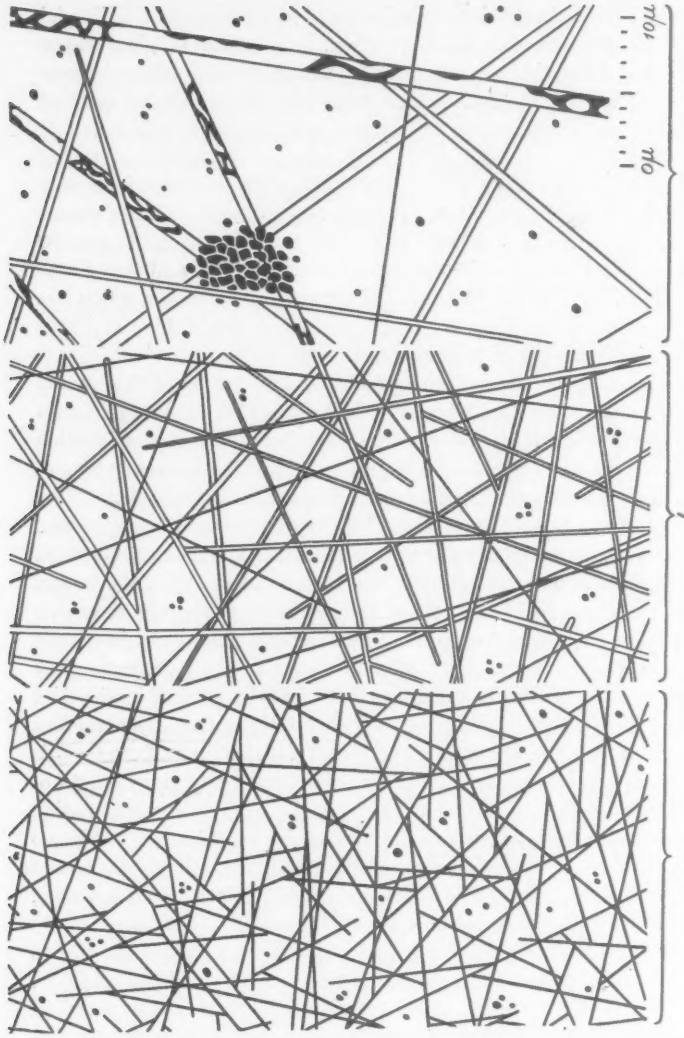


Fig. 3. Fibril formation at junction of droplets of (1) citrated human blood plasma and (2) citrated solution of venom of *Echis carinatus* (1 in 8,000,000). Film preparation six days old. *a* is taken at the venom side of the junction of liquids; *b* at the centre; and *c* at the plasma side. In *a* the fibrils are fine and numerous; in *b* most of the fibrils are larger, though less numerous; in *c* the fibrils are still further reduced in number, a few of relatively considerable size exhibiting a reticular pattern. Highly refractile dancing particles are seen among the fibrils: in *c* a fixed collection of particles attached to fibrils is observed. ×2300.

Exp. B. A film preparation was made by placing droplets of human blood plasma and of a 1 in 8,000,000 solution of venom of *Echis carinatus* (both liquids being citrated) respectively near each other on a glass slide, within an area ringed with vaseline. A coverslip was then applied and lightly pressed down, so as to allow the droplets to come in contact at their opposed edges with little or no mixing of the liquids, evaporation being prevented by the ring of vaseline. The appearance of the junction of liquids at the end of six

days is shown in Fig. 3. At the side of the junction near the venom solution (*a*) very fine, exceedingly numerous fibrils are seen; in the middle (*b*) larger, less numerous fibrils; at the side next the fibrinogen solution (*c*) only relatively few fibrils, mostly long, of large diameter, and exhibiting an irregular pattern are observed.

Fibrin fibrils it may be observed are usually found to be unequal not only in length but also in thickness. After the lapse of a fortnight or three weeks, when they appear to have reached their maximum thickness, it may be observed that, mixed with the largest fibrils, medium-sized and fine fibrils are also seen (Fig. 3 *c*); when the fibrils do not exceed a medium value fine fibrils may be seen as well (Fig. 3 *b*); but when liminal visibility of fibrils has been reached differences of thickness can no longer be recognised.

It will be noted that those fibrils which are situated near the venom solution are contained in a fluid which is relatively rich in venom and correspondingly poor in fibrinogen, while at the opposite aspect of the junction of liquids the fibrils are present in a liquid in which the concentration of fibrinogen reaches its maximum, the venom being exceedingly dilute.

In order to observe more fully the effect of dilution of fibrinogen and venom respectively the series of experiments in Tables I and II were carried out. In these experiments an attempt has been made to give the actual number of fibrils observed in the field of the microscope: the higher counts are only approximate, owing to the large number and fineness of the fibrils present; the lower counts are more reliable owing to the smaller number and larger size of the fibrils. The enumeration of fibrils present in the field of the microscope is attended with difficulty if the fibrils are very numerous or are exceedingly fine. In the former case (*cp.* Fig. 3 *a*) delimitation of fibrils cannot be effected: it is a matter of uncertainty whether any given length of fibril should be regarded as a separate fibril or is merely the continuation of an adjacent fibril. Again, when fine fibrils are being examined, it is difficult to avoid overlooking some of the finest, which are indeed barely visible even with the maximum intensity of illumination available. It is obvious, therefore, that, if the fibrils under observation are very numerous or are exceedingly fine, enumeration is merely approximate. On the other hand if the conditions of experiment are such that the fibrils are few in number and if, in addition, they are relatively thick, counting ceases to be difficult and accuracy of enumeration is attainable. In Table I the effect of an 81-fold dilution of venom is exhibited. It will be seen that, within the range of concentration employed, the number of fibrils visible appears to be roughly proportional to the amount of venom present. This relation, it may be observed, does not continue to hold if the range of concentration of venom is made considerably more extensive: under these circumstances a point is reached at which no further increase occurs, but an actual diminution in the number of fibrils recognisable may be noted (*cp.* p. 209). With the highest venom concentration employed

in Table I, fibril formation is not only abundant but also rapid, occurring within a few minutes, while with the lowest concentration its course is very slow, fibrils not appearing until after the lapse of many hours: in the former case only a limited increase in thickness of the fibrils subsequently occurs; in the latter the amount of fibrinogen available is, owing to the smaller number of fibrils, relatively considerable and in consequence marked though slow growth takes place, which may not reach its maximum for several days.

Table I.

Equal parts of citrated human plasma (undiluted) and of citrated venom solution, the latter in varying dilutions, were placed on a glass slide and, immediately after mixing, a coverslip applied, the thickness of fluid examined being 0·05 mm.

Venom	Fibrils per field	Appearance of fibrils
in 300,000	1730	Very fine, retracted
„ 900,000	640	Fine
„ 2,700,000	120	Medium
„ 8,100,000	50	Moderately coarse
„ 24,300,000	20	Coarse

In Table II the effect of dilution of plasma is exhibited: it is seen that, with diluted plasma, the number of fibrils diminishes, but the diminution is no longer accompanied by an increase in thickness, as in the preceding experiments, the fibrils remaining fine. With the higher dilutions no fibrils can be recognised. Fibrils make their appearance in diluted plasma more slowly than in undiluted plasma.

Table II.

Equal parts of citrated human plasma, in varying dilutions, and of citrated venom, diluted 1 in 8,100,000, were mixed together as in the preceding Table.

Plasma	Fibrils per field	Appearance of fibrils
100 %	43	Moderately coarse
33	7	Fine
11	—	No fibrils visible
3·7	—	„ „
1·2	—	„ „

Returning once more to the appearances presented by fibrin fibrils at the junction of two liquids containing respectively plasma and venom (*Exp. B*, p. 193, and Fig. 3 *a*, *b*, *c*), we are now, by the aid of the data obtained from the above dilution experiments, in a position to state that at the venom side of the junction of liquids (where venom is relatively abundant and fibrinogen dilute) the numerous fine short fibrils observed have been produced rapidly, while at the fibrinogen side of the junction (where plasma is almost undiluted and venom is highly dilute) the long, relatively coarse fibrils, which are seen in comparatively small numbers, have been produced with considerable slowness.

This mode of formation of fibrin fibrils in the presence of varying concentrations of venom indicates the source of the particles of origin of the fibrils.

These particles of origin, which, it may be surmised, are contained either in the thrombin solution or in the fibrinogen solution, employed for fibril production, may conceivably consist of either thrombin or fibrinogen. If the particles of origin consist of thrombin the effect of dilution of the thrombin solution, the concentration of fibrinogen remaining unchanged, would be to diminish the number of fibrils without affecting their time of formation. Such, however, is not the case: when the concentration of thrombin is lowered fibrils make their appearance (in diminished numbers) after a lengthened interval, the duration of which is dependent upon the degree of dilution of thrombin. The hypothesis that the particles of origin of fibrin fibrils consist of thrombin is therefore untenable. If, on the other hand, the particles of origin consist of fibrinogen the effect of dilution of thrombin, acting catalytically, would be exhibited by delay of formation of fibrils, and if the particles of origin were unequal (for example, in consequence of variability in size, as is not inherently improbable) in respect of their response to thrombin, the fibrils appearing would be reduced in number: since both of these effects are actually observed it is seen that the latter hypothesis, namely that the particles of origin of fibrin fibrils consist of fibrinogen, is tenable in so far as it is consistent not only with the delay in appearance of the fibrils but also with the diminution of their number[1]. Further experimental proof of the validity of this hypothesis will now be given.

Fibrinogen solution.

Since growth of fibrils can be observed in a fibrinogen solution the further assumption must be made that, while part of the fibrinogen is in suspension, another part is in solution: in other words the liquid containing fibrinogen must be diphasic, forming a hydrosol, the disperse phase furnishing the particles of origin of the primitive fibrils, while the continuous phase, by affording a further supply of fibrinogen to the primitive fibrils, enables an increase of their diameter to occur, both particulate and added fibrinogen alike changing under the influence of thrombin into fibrin.

A point has now been reached at which it is possible to put hypothesis to the test of experiment. If fibrinogen solution forms a two-phase system the volume of the concentrated phase which, it has just been inferred, provides the particles of origin of fibrils will diminish on dilution, that of the dilute phase undergoing a corresponding increase, until ultimately a dilution is reached, below which the concentration of the dilute phase, previously maintained unchanged, will now begin to diminish with further addition of diluting fluid.

In order to test this conclusion an attempt was made to determine the concentration of the continuous phase present in varying dilutions of plasma.

[1] The effect of dilution of fibrinogen solution upon the thickness of fibrils is readily explicable The latter in low concentrations of fibrinogen can receive only a small supply of this substance and in consequence remain exceedingly small.

To this end the experiments on fibril formation, shown in Table III, in which the time of first appearance of fibrils in a series of mixtures of fibrinogen and venom is observed, were made. This method of enquiry is based upon the assumption that, since one of the two substances interacting in the production of fibrils, namely venom, is employed in the same concentration in all experiments, fibril formation will therefore proceed at a monomolecular reaction rate according to the equation

$$-lC = kt + a,$$

where C = concentration of continuous phase, t = time of first appearance of fibrils, and k and a are constants. If this assumption is verified it would become possible to determine the degree of dilution of plasma below which diminution of concentration of the continuous phase commences, and to ascertain the relative concentration of continuous phase corresponding to any given dilution of plasma. The value of the constant k is independent of that of a: if the temperature of experiment is maintained invariable its value is dependent upon the concentration of thrombin employed. If the curve is made to commence at the axis of ordinates so that the vertical part corresponds with this axis, then $-a$ represents the natural logarithm of the lowest concentration of plasma at which the continuous phase is saturated.

Table III.

Citrated human plasma, in varying dilutions, was mixed with citrated venom, 1 in 30,000, in the proportion of eight parts of the former to one part of the latter, and the time at which fibrin fibrils were first observed noted.

Exp.	Plasma	Fibrils first seen at end of:
1	100 %	5 minutes
2	90 „	6 „
3	80 „	5 „
4	70 „	7 „
5	60 „	12 „
6	50 „	50 „
7	40 „	113 „
8	30 „	175 „
9	20 „	262 „
10	10 „	No fibrils observed

The observations when plotted with dilution of plasma as ordinates and time of first appearance of fibrils as abscissae were found to describe a curve (Fig. 4 *a*) so long as the concentration of plasma employed did not exceed 61 % (undiluted plasma[1] being represented by 100): above this point the curve was replaced by a vertical line. The curve corresponds to the concentration of the continuous phase, which reaches its maximum at a dilution of

[1] The precipitate (dried to constant weight at 120°) obtained from plasma by adding twenty volumes of distilled water, through which a stream of carbon dioxide had been passed, amounted to 0·8 % of the plasma employed. This precipitate is known to contain, in addition to fibrinogen, prothrombin and thrombokinase. It is not at present possible to obtain fibrinogen in even an approximately pure condition.

plasma represented by 61 %: at this point the conversion of the disperse phase into the continuous phase is apparently complete. Below a dilution of 61 % the concentration of the continuous phase becomes lowered when more fluid

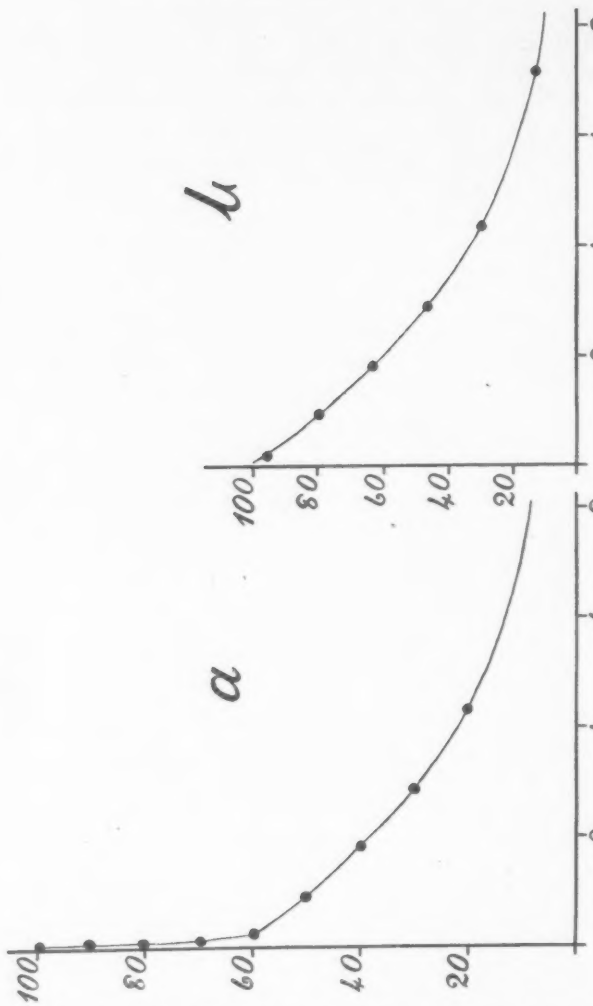


Fig. 4 *a* and *b*. Graphic representation of experiments in Table III. Ordinates in Fig. 4 *a* indicate the total concentration of fibrinogen contained in the plasma employed, that of undiluted plasma being represented by 100; ordinates in Fig. 4 *b* give the concentration of fibrinogen in the continuous phase, that corresponding to 61 % in Fig. 4 *a* being here represented by 100. Abscissae represent time, in hours, at which fibrin fibrils were first observed. The curves are constructed from values of $k = 0{\cdot}0042$ and $a = -4{\cdot}623$. Exps. 1 to 4 are not indicated in Fig. 4 *b*.

is added; above this dilution no further change in the concentration of the continuous phase occurs, saturation having been attained. In Fig. 4 *b*, the same curve is again given the ordinates now indicating the concentration of the continuous phase, saturation of which (corresponding to 61 % of plasma

in Fig. 4 *a*) is represented by 100. If the values of the constants, determined from *Exps.* 5 to 10 (Table III), are taken at

$$a = -4{\cdot}623, \qquad k = 0{\cdot}0042,$$

the calculated reaction times, given in Table IV, exhibit little divergence from those actually observed. From these experiments it may therefore be concluded that fibrinogen solution is diphasic. That the individual particles of the disperse phase have not, however, completely disappeared when the transition point of the curve is reached, is shown by the circumstance that fibril formation does not cease at this point. Nevertheless the sharpness of the transition point and the fair correspondence of calculated and observed times appear to indicate that the amount of fibrinogen still remaining, with increasing dilution of plasma, in the particles of the disperse phase after the maximum concentration of the continuous phase has been passed, is relatively insignificant, and thus the assumption, made in Table IV, col. 2, that the concentration of the latter phase now becomes proportional to the further dilution appears to be at any rate approximately correct. The significance of the absence of complete disappearance of the particles of the disperse phase, at the transition point, will be referred to later (p. 208).

Table IV.

Calculated time of appearance of fibrin fibrils in the experiments recorded in Table III.

Exp.	Plasma	Fibrinogen in continuous phase[1]	Reaction calculated	Time observed
1	100 %	100 %	5·5 min.	5 min.
2	90 „	100 „	5·5 „	6 „
3	80 „	100 „	5·5 „	5 „
4	70 „	100 „	5·5 „	7 „
5	60 „	98·5 „	10 „	12 „
6	50 „	82·0 „	53 „	50 „
7	40 „	65·6 „	107 „	113 „
8	30 „	49·2 „	178 „	175 „
9	20 „	32·8 „	271 „	262 „
10	10 „	16·4 „	437 „	No fibrils observed

If the dilution of plasma is considerable no visible fibrils are obtainable on adding thrombin: in Table II, for example, this was found to be the case with a dilution of 11 % of plasma. When, however, a portion of this highly diluted plasma was placed on a microscope slide and allowed to evaporate at room temperature to about one-sixth of its bulk, and then a small amount of venom (1 in 30,000) added, a striking result was obtained: at the end of fifteen minutes abundant fibril formation was found to have occurred; in a control experiment, performed at the same time, in which care was taken to prevent evaporation, no fibrils appeared.

It is thus evident that the simple addition of thrombin as a test for fibrinogen under all circumstances is not admissible. If the continuous phase is sufficiently dilute (*cp.* p. 203), no coagulation is recognisable, no fibrils

[1] The maximum concentration, which is present in 61 % dilution of plasma, being represented by 100.

are observable: by evaporation, however, the original concentration of fibrinogen may be regained and the former coagulability reproduced.

Incidentally the ease with which a highly diluted continuous phase can be concentrated, with reappearance of visible fibrils, in experiments made with small quantities of diluted plasma, placed on a glass slide, emphasises the necessity of very carefully avoiding evaporation in such experiments when determinations of the time of first appearance of fibrils are being made.

Mechanism of formation of Fibrin Fibrils.

With a view of throwing further light upon the processes concerned in coagulation, the following hypothesis as to the mechanism of production of fibrin fibrils may now be advanced.

In attempting to explain the mode of occurrence of this phenomenon in a liquid containing fibrinogen and thrombin I picture to myself fibrils originating from spherical particles, exhibiting Brownian movement like the particles ordinarily seen in plasma, but presumably of considerably smaller dimensions, which under the influence of thrombin have become semi-solid, viscid and sticky. One of these oscillating particles comes, let us suppose, into contact with the surface of the glass slide, upon which the mixture of fibrinogen and venom solutions, used for experiment, has been placed. Recoil then occurs, the particle changing its direction, continuing its oscillatory movement, and ultimately, after an exceedingly zig-zag course, colliding a second time with, let us imagine, the glass slip covering the fluid in which it is contained. But inasmuch as the particle is viscid and sticky it became adherent to the glass slide at the first impact and then, upon recoil, became drawn out, so that when the second collision with the cover-slip occurred a primitive fibril had thus been formed, which now became adherent by its previously free end (containing the remainder of the particle of origin) to the cover-slip, the fibril being thus attached at each extremity to a glass surface. By the continued action of thrombin the further change of the primitive fibril, thus formed, into fibrin is completed: the fibril becomes solid and, at the same time undergoing contraction, becomes also tense. Further addition of fibrinogen, from the continuous phase, to the primitive fibril next occurs, the new supply becoming in its turn, under the influence of thrombin, converted into solid fibrin. In this way a relatively thick fibril, visible under ultramicroscopic illumination, is ultimately produced. Adhesion of the primitive fibrils is not limited to glass surfaces. Attachment to other fibrils is common, especially when the latter are abundant, and adhesion to the particles seen singly or in groups in fibrinogen solution is also frequently observed[1].

[1] The latter in many cases seems, however, to be a late event, accompanying and partly dependent upon gradual subsidence of the particles under the force of gravity. Characteristic rosette figures, consisting of fibrils radiating from a group of granules, may thus be formed.

Both particles of origin and primitive fibrils appear to be amicroscopic (*cp.* p. 205).

Further Characters of Fibrinogen.

In relation to fibril formation the processes occurring prior to the first appearance of visible fibrils appear to be, as already indicated, three in number: (1) change, under the influence of thrombin, of the particle of origin, consisting of fibrinogen in a concentrated condition, presumably forming a liquid droplet, into a viscid, sticky, semi-solid mass; (2) conversion of the latter, by virtue of its Brownian movement, leading to contact with neighbouring surfaces, into a primitive fibril stretching between two surfaces to which its ends adhere, and changing under the further action of thrombin into fibrin; (3) growth of the fibril by deposition of fibrinogen from the continuous phase, the fibrinogen so deposited being changed in its turn, by the thrombin present, into fibrin. In the series of experiments, made to determine the time of first appearance of visible fibrils, given in Table III and Fig. 4, the period occupied by the first two processes must be alike in all experiments, whatever the dilution of fibrinogen may be, since the initial concentration of thrombin is the same throughout the series. This period was estimated for the dilution of plasma corresponding to *Exp.* 5, Table III, in the following way. As soon as the mixture of fibrinogen and thrombin had been prepared it was stirred continuously for five minutes, portions being removed for examination at the end of one, two, three, four and five minutes respectively, and the approximate number of visible fibrils appearing in a fixed volume of the sample determined. Such primitive fibrils as were formed before taking the sample would have been already removed by stirring; the visible fibrils observed in the samples must, therefore, indicate primitive fibrils formed after removal and would correspond to particles of origin which had not been converted into primitive fibrils at the time of removal of the sample. The observations made are given below:

Time	Fibrils		
	observed	calculated	%
0 min.	—	732	100
0·07 „	—	659	90
1 „	135	147	20
2 „	33	30	4
3 „	5·5	5·9	0·8
4 „	in exceedingly scanty numbers	1	0·2
5 „	„	0·2	0·03

These experiments, which are represented graphically in Fig. 5, indicate that the change of particles of origin into primitive fibrils proceeds at a monomolecular reaction rate, the constants of the reaction being $k = 1{\cdot}6$ (time being reckoned in minutes) and $a = -6{\cdot}58$: the calculated number of fibrils, given in the third column (and in percentage form in the fourth), agree with the observed values within the range of experimental error. The time occupied in the formation of primitive fibrils appears to be small: according to the above

calculation four-fifths of the maximum number of fibrils obtainable are formed within one minute of mixing fibrinogen and thrombin, and one-tenth within the first four seconds (0·07 min.)[1]. On the other hand it will be noted that in the experiments recorded in Table III, in which the same concentration of thrombin was used as in the observations given above, the minimum time of first appearance of visible fibrils was five minutes. Since the fibrils first appearing form less than one-tenth of those ultimately seen it is clear that the reaction actually measured in the experiments recorded in Table III is essentially the process represented above by (3), namely growth of primitive fibrils, the processes (1) and (2) together occupying a negligible fraction of the period of first appearance of visible fibrils.

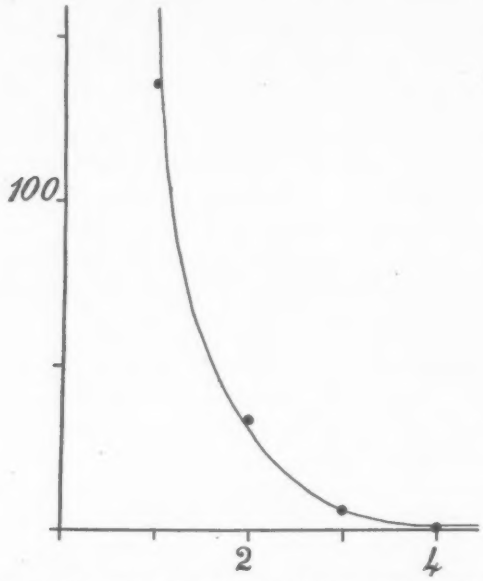


Fig. 5. Removal of primitive fibrils from mixture of fibrinogen and thrombin by stirring with a glass rod. Ordinates represent the number of fibrils appearing in samples removed after varying periods of stirring; abscissae represent time in minutes. Details of experiments are given in text. The curve is constructed from values of $k = 1{\cdot}6$ and $a = -6{\cdot}58$.

It will be seen from Tables III and IV that fibrils may be formed, not only in undiluted plasma (*Exp.* 1), but also when only 20 % of plasma is present (*Exp.* 9). In the former experiment the maximum concentration of both disperse and continuous phases obtains; in the latter the disperse phase has nearly completely disappeared and the continuous phase has only 33 % of its maximum value (transition point of curve at 61 % of plasma). If an

[1] In mixing fibrinogen and thrombin solutions stirring should be as far as possible avoided in experiments in which fibril counting is attempted.

endeavour is made to count the fibrils present[1], as in the experiments recorded in Table II, it is found that the approximate number present does not at first exhibit any marked decrease, but the fibrils become very fine and when dilution of the continuous phase is considerable cease to be visible owing to their extreme tenuity. Below a series of experiments performed under the same conditions as in Table III (the concentration of thrombin being constant, while fibrinogen is employed in varying degrees of dilution) is given, in which an attempt is made to enumerate the fibrils present in a given small volume of each mixture of fibrinogen and thrombin. These results are plotted in Fig. 6. The higher

Citrated plasma	Fibrils
100 %	106
85 „	130
70 „	113
60 „	118
50 „	114
40 „	80
30 „	33
20 „	0·2
10 „	0

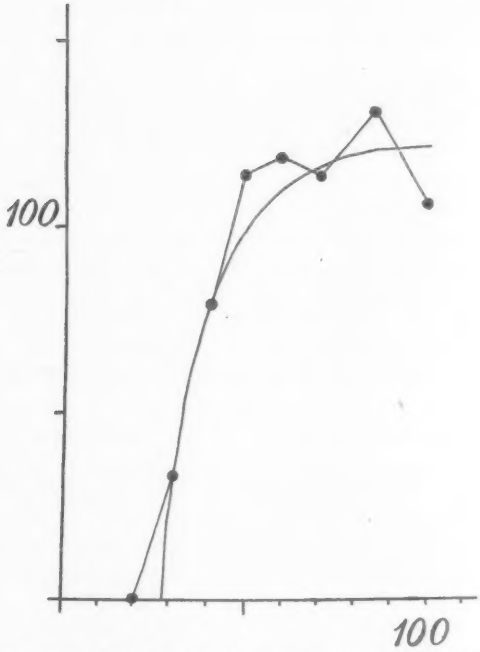


Fig. 6. Approximate number of fibrils obtained on coagulation of varying dilutions of citrated human plasma. Ordinates represent fibrils; abscissae represent percentage of plasma. Details of experiments are given in text. The fibril counts indicate a curve of the type shown.

[1] The enumeration is approximate: the error is considerable when fibrils are abundant.

counts are only approximate, owing to difficulties already referred to, but the type of curve formed by the fibrils is indicated in the figure: the dilution of plasma at which visible fibrils cease to be obtainable is determined graphically to correspond to 28 % of plasma. Since the maximum concentration of the continuous phase of fibrinogen in these experiments was found to be present in 75 % of plasma and in all concentrations of plasma above this point, it follows that, in this series of experiments, fibrils cease to be visible when the continuous phase is reduced to 37 % of its saturation concentration. This result and those of three other similar determinations are given below:

Series	Continuous phase of fibrinogen
A	26 %
B	30 „
C	40 „
E	37 „

The mean of the series corresponds to 33 % of the maximum concentration of the continuous phase.

The action of thrombin in converting fibrinogen in the concentrated phase into fibrin is not exerted solely upon the dispersed particles of this phase, as is shown by the following experiment.

Exp. C. Precipitated fibrinogen, obtained by adding to plasma twenty volumes of distilled water through which a stream of carbon dioxide had been passed, was suspended in distilled water so as to form a thick emulsion, composed of closely packed masses of fine motionless granules, 0·4μ or less in diameter. This emulsion was divided into two parts, one of which (*B*) was used for control experiments; to the other venom, contained in distilled water, was added, the concentration of venom in the mixture being about 1 in 1,000,000. At the end of forty-eight hours the mixture had formed a soft, semi-solid, friable mass, consisting of densely packed fine granules free from fibrils: this was broken up as completely as possible and suspended in distilled water (*A*). Portions of *A* and *B* were used for experiment as follows:

	1 part	+4 parts citrated 0·85 % NaCl containing	Microscopic appearance with dark ground illumination
1.	*A*	—	No dispersion. No fibrils.
2.	*A*	venom 1 in 30,000	Marked dispersion. No fibrils.
3.	*A*	„ 2·3 %	Nearly complete dispersion. No fibrils.
4.	*B*	—	Complete dispersion. No fibrils.
5.	*B*	venom 1 in 30,000	Complete dispersion. Fibrils fairly numerous.

The last observation was repeated in a small test-tube: a loose coagulum formed, which had already retracted at the end of three hours.

In the above experiment it is seen that under the action of thrombin precipitated particles of fibrinogen are converted into fibrin without any

change of form, fibril formation being impossible owing to the immobility of the particles. The particles of fibrinogen, however, adhere to each other and to the numerous adventitious particles which accompany the precipitated fibrinogen, so that the latter cannot be dispersed on adding 0·85 % sodium chloride solution. If, however, the sodium chloride solution contains venom in sufficient concentration to dissolve the altered fibrinogen particles, then dispersion occurs. That the fibrinogen is converted into fibrin is indicated by the effect of relatively concentrated venom in producing lysis (see p. 209). It does not appear that the presence of sodium chloride is necessary for the transformation of fibrinogen into fibrin. Direct conversion of some of the dispersed particles of fibrinogen in the concentrated phase does not seem inherently improbable, but investigation of this point is attended with difficulty owing to the presence of numerous adventitious dancing particles in liquids containing fibrinogen.

That the suspended particles of the concentrated phase of fibrinogen are unequal, presumably, in size is shown by the circumstance that (1) primitive fibrils are formed at different periods of time (p. 201), while visible fibrils (2) are of different size (Figs. 1–3), (3) do not all appear at the same time (p. 190), and (4) are reduced in number when highly dilute thrombin is employed (Table II; also p. 202).

The size of the particles which give rise to primitive fibrils does not appear to be capable of measurement, nor can the diameter of primitive fibrils be at present determined, but it is nevertheless possible to give, for both of these, upper limits of size, which cannot be exceeded. Thus the finest fibril which could be recognised with the illumination employed in these experiments had an apparent diameter of about 0·15μ. Assuming the length of such a fibril to be 50μ, its volume would be represented by a sphere of 0·46μ radius. Such a relatively large particle, however, though possessing oscillatory movement, would be much too defective in translatory motion to give rise to a primitive fibril 50μ long. On the other hand the smallest recognisable particle, having an apparent diameter of 0·15μ, though still possessing insufficient translatory movement, would have the same volume as a fibril 50μ long and 0·0067μ in diameter. These two values, therefore, may be taken to represent maximum limits for the diameter of a particle of origin and for the thickness of a primitive fibril respectively.

The very irregular translatory movement of an oscillating particle having an apparent diameter of 0·15μ did not, as a rule, exceed 10μ in the course of a minute. Since fibrin fibrils may reach 400μ or more in length and more than four-fifths of the primitive fibrils may be already formed within a minute of the addition of thrombin (p. 201), it follows that the average size of the particles of origin of the fibrils must be much less than that of the smallest visible particles observable in these experiments. Several observers have attempted to measure the mean amplitude of oscillation and degree of translation of particles of known diameter [*cp.* R. Zsigmondy, 1905, pp. 104–111],

but sufficient data do not appear to be yet available to enable the limits of size of such colloidal particles of fibrinogen as possess enough translatory motion to be capable of giving rise to primitive fibrils to be determined.

The maximum or saturation concentration of the continuous phase of fibrinogen in plasma obtained from the same source must be assumed to be constant under the same conditions of experiment (concentration of thrombin and temperature) since the time of first appearance of visible fibrils remains unchanged[1]. It is therefore possible, when the transition point of the reaction rate curve has been determined (*cp.* Table IV, Fig. 4 *b*), to express the concentration of the disperse phase in terms of the saturation concentration of the continuous phase. If this is done in respect of the same sample of plasma at different periods after collection, it is found that the disperse phase of fibrinogen gradually diminishes in amount on keeping. This disappearance of the disperse phase is exhibited in the following series of experiments each series being carried out as indicated in Table III:

Series	Age of plasma	Concentration of disperse phase of fibrinogen		
		observed	calculated	in percentage form
—	0 days	—	219	100
A	6 „	163	162	74
B	12 „	120	120	55
C	18 „	92	89	41
D	25 „	64	63	29
E	39 „	33	31	14

In the second column the period after bleeding, at which the experiments were made, is shown; in the third column the concentration of the disperse phase is given in terms of the continuous phase, represented by 100[2]; in the fourth column these concentrations (and also the initial concentration) are calculated on the assumption that the change of the disperse phase proceeds at a logarithmic rate, the constants of the reaction being estimated at $k = 0{\cdot}05$ (time being measured in days) and $a = -5{\cdot}38$; in the fifth column the concentrations given in the fourth are presented in percentage form. The close correspondence between observed and calculated values, exhibited graphically in Fig. 7, establishes the truth of the assumption that the disappearance of the disperse phase proceeds at a monomolecular reaction rate. This occurs equally when the colloidal particles of this phase are suspended in undiluted or in diluted plasma, thus being independent, within the limits of experiment, of the dilution. The continuous phase of fibrinogen does not appear to be affected by the addition of thrombin: if any disappearance of this phase occurred it

[1] In Series *B*, *C*, *D*, the same stock solution of thrombin (kept at 0°) was employed: the time of first appearance of visible fibrils in undiluted plasma or in plasma dilutions lying above the transition point of the corresponding curve was 8·5 minutes. In Series *A* and *E* in which freshly prepared thrombin solutions were used this period was slightly reduced.

[2] In the series of experiments given in Table III, for example, the concentration of the disperse phase would be represented by $\frac{39 \times 100}{61} = 63{\cdot}9$.

would presumably proceed at a constant rate in all concentrations of plasma above the transition point, in other words so long as the maximum concentration of the continuous phase is maintained, but of this there is no evidence in experiments of the type of those given in Table III.

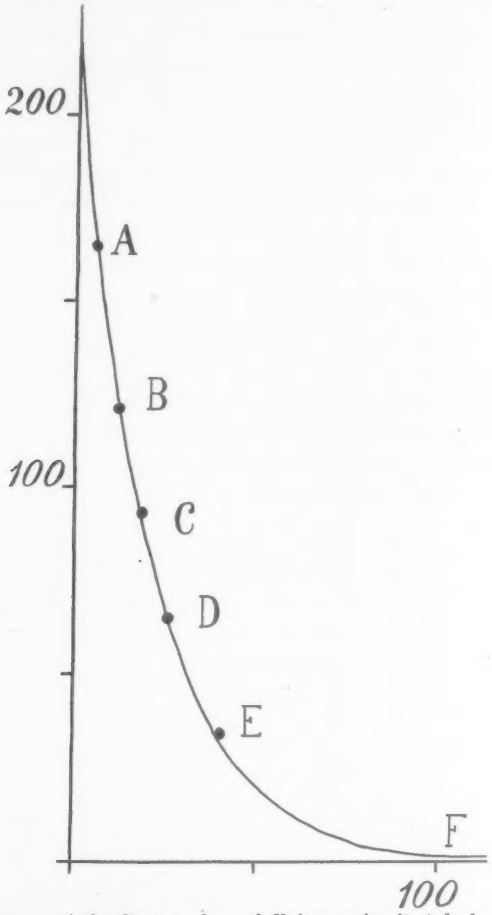


Fig. 7. Disappearance of the disperse phase of fibrinogen, in citrated plasma, on keeping. Ordinates represent concentration of disperse phase in terms of maximum concentration of continuous phase, represented by 100; abscissae represent time in days. Details of experiments are given in text. The curve is constructed from values of $k = 0{\cdot}05$ and $a = -5{\cdot}38$.

The disappearance of the disperse phase on keeping is in part, if not wholly, due to fibril formation, for when this disappearance is considerable, flocculent masses, which are found on microscopical examination to consist of radiating collections of coarse fibrils mixed with aggregations of granules,

are observed in the plasma, which has become very turbid though still liquid. Whether in addition any of the particles of the disperse phase, in consequence, it may be, of their relatively large size or of an accidental failure to collide with a surface before conversion into fibrin was complete, become directly converted into fibrin is uncertain, the presence of numerous dancing particles, as already pointed out, making it difficult to determine this point.

The particles of the disperse phase, so long as the change of fibrinogen into fibrin is incomplete, consist of a mixture of fibrin and fibrinogen. If neither of these substances is soluble in or miscible with the other no explanation is forthcoming of the failure of the disperse particles to disappear completely at the transition point of the curve, nor is the growth of primitive fibrils accounted for. If, however, it is assumed that fibrin, or any product intermediate between fibrin and fibrinogen, is soluble in fibrinogen in the disperse phase, then the concentration of fibrinogen in disperse particles in which partial change into fibrin had occurred would be lowered and would no longer be in equilibrium with the continuous phase when the latter was saturated. In consequence of this disturbance of equilibrium fibrinogen would pass from the continuous phase into the particles of the disperse phase (or into the primitive fibrils, if the particles had already given rise to fibrils). In this way a new equilibrium between the disperse phase (or primitive fibrils) and the continuous phase would be reached with increasing dilution of the latter. As more of the disperse phase (or primitive fibrils) became converted, under the further action of thrombin, into fibrin this new equilibrium would, in its turn, be disturbed and a further transfer of fibrinogen from the continuous phase into the disperse particles (or fibrils) would occur, these processes continuing until finally all the fibrinogen had been converted into fibrin.

The above appears to be the simplest hypothesis which will account both for the absence of complete disappearance of disperse particles of fibrinogen at the transition point of the reaction rate curve (Fig. 4) and also for the growth of primitive fibrils: it may therefore on this account be, provisionally at any rate, adopted. Two corollaries, it may be pointed out, follow from this hypothesis. The first is that the concentration of the continuous phase below the transition point is not strictly proportional to the further dilution: since, however, the amount of fibrinogen present in the disperse phase, when determined by experiments of the type of those shown in Table III, appears to be very small, it may be surmised that the difference in question is also inconsiderable. The second corollary is that if any of the disperse particles are too large to possess the degree of translatory movement necessary for their conversion into primitive fibrils, undergoing in consequence direct conversion into fibrin, such particles must also subsequently increase in size at the expense of fibrinogen present in the continuous phase, in the same manner as primitive fibrils.

When relatively concentrated venom of *Echis carinatus* is employed as source of thrombin a further action occurs: the fibrils which make their

appearance subsequently disappear, undergoing lysis[1]. Thus a 1 in 3000 solution of venom, added to an equal volume of undiluted plasma, leads to abundant fibril formation in about three minutes. At the end of two hours at room temperature (16°) most of the fibrils have disappeared, those remaining being thinned and for the most part broken, forming débris which falls to the bottom of the layer of fluid examined and is later completely dissolved. The occurrence of this lysis in particles of precipitated fibrinogen, which have undergone direct conversion into fibrin, has been already described (p. 204). On the hypothesis that lysis of fibrin is not due to an adventitious proteolytic ferment, but is a true thrombin effect, the change of fibrinogen in the concentrated phase into fibrin, under the action of thrombin, would appear to be merely a preliminary stage of the lytic action of thrombin.

Bearing on gel formation in general.

The behaviour of fibrinogen under the action of thrombin suggests that in any colloidal solution the disperse phase, if it consists of liquid particles, may, when passing into the solid state under the influence of a fall of temperature or of ferment action, give rise to fibrils, if the particles are sufficiently small, or form a suspension of solid particles, if of relatively large size. In this way may be explained the formation of the characteristic structure, consisting of a mixture of network and granules, sometimes the one sometimes the other predominating or being present exclusively, met with in cell plasm, subjected to the action of fixatives [see Wilson, 1904], and also in gels after similar fixation [Hardy, 1899].

Summary.

1. Fibrinogen in solution is diphasic, consisting of a concentrated phase, which is dispersed, and a dilute phase, which is continuous.

2. Under the action of thrombin fibrin is formed out of fibrinogen in the concentrated phase: if this phase is in the form of a precipitate its particles become directly converted into fibrin; if this phase is dispersed its suspended particles give rise to fibrin fibrils, forming a coagulum.

3. The action of thrombin upon fibrinogen in the concentrated phase is purely catalytic. No effect appears to be exerted by thrombin upon fibrinogen in the continuous phase.

[1] Cp. Houssay and Negrete [1918]. These authors observe that solid proteins with the exception of fibrin are not dissolved: the action of venom thus appears to be specifically directed to fibrin.

4. The following hypothesis of the mode of formation of fibrin fibrils is advanced:

Fibrin fibrils are particulate in origin.

The particles of origin, consisting of fibrinogen in relatively high concentration, contained in a solution of fibrinogen of low concentration, and possessing oscillatory and translatory movement, (1) become, under the action of thrombin, semi-solid, viscid and sticky, and (2) form, as the result of collisions, adhesions to solid surfaces, with the result that they (3) become drawn out into primitive fibrils.

Increase in thickness of the primitive fibrils takes place by addition of fibrinogen, derived from the solution of fibrinogen of low concentration.

5. When fibrinogen in the concentrated phase has become partially changed into fibrin, the concentration of fibrinogen in this phase is lowered, disturbance of equilibrium of the two phases resulting, and fibrinogen passing from the dilute into the concentrated phase. In this way may be explained the "growth" of fibrin fibrils. This growth is observed to occur at a monomolecular rate.

6. If, as source of thrombin, venom in sufficient concentration is used, the fibrin formed, whether consisting of fibrils or of particles of fibrinogen in the concentrated phase which have been directly transformed into fibrin, later undergoes lysis.

7. The action of thrombin upon fibrinogen affords an explanation of the mechanism of production of the reticular structure observed in gels and cell plasm.

REFERENCES.

Barratt (1915). *Proc. Roy. Soc.* B. **87**, 179.
Cesana (1908). *Arch. Fisiol.* **5**, 180.
Hardy (1899). *J. Physiol.* **24**, 158.
Houssay and Negrete (1918). *Revista del Instituto Bacteriologico del Departamento Nacional de Higiene*, Buenos Aires, **1**, 341.
Howell (1910). *Amer. J. Physiol.* **26**, 253.
—— (1914). *Amer. J. Physiol.* **35**, 143.
—— (1916). *Amer. J. Physiol.* **40**, 526.
Mayer (1907). *Compt. rend. Soc. Biol.* **63**, 658.
Stübel (1914). *Pflüger's Arch.* **156**, 361.
Wilson (1904). *The Cell* (London, Macmillan).
Zsigmondy (1905). *Zur Erkenntnis der Kolloide* (Jena).

XXIII. EFFECT OF HEAT ON THE ANTI-SCORBUTIC ACCESSORY FACTOR OF VEGETABLE AND FRUIT JUICES.

By ELLEN MARION DELF.

From the Department of Experimental Pathology, Lister Institute.

(*Received March 2nd, 1920.*)

A KNOWLEDGE of the properties of the accessory food factors is of especial importance in connection with the cooking of food and with its successful preservation. In the case of the best known accessory factors—the water-soluble, the fat-soluble and the anti-scorbutic vitamines—the effect of exposure to heat for shorter or longer periods is not similar, but comparatively little quantitative work has been done on these lines.

The water-soluble factor is now commonly identified with the substance which has been shown to cure polyneuritis of pigeons. Chick and Hume [1917] were unable to detect that the anti-neuritic factor present in wheat germ and in yeast extract was diminished after exposure to a temperature of 100° for two hours, but a temperature of 113° prolonged for 40 minutes destroyed about half the activity of these substances, the rate of destruction being increased at higher temperatures. More recently it has been asserted by McCollum and Simmonds [1918] that the water-soluble vitamine extracted from "navy beans" is destroyed if heated for an hour at 100° in an alkaline medium (0·28 % NaOH).

The fat-soluble vitamine is more sensitive to heat but the evidence on this point is not yet conclusive. According to Steenbock, Boutwell and Kent [1918], butter fat loses nearly all its growth-promoting power after being heated for 4 hours at 100°, and Drummond detected some loss in value after 1 hour's heating at 100° [1919]; moreover the hydrogenation of whale oil at high temperatures completely destroys the fat-soluble content of the original oil [Halliburton, 1919]. On the other hand, the results of numerous observations by workers at the Lister Institute upon milk heated in an autoclave at 15 lb. pressure (*i.e.* at about 120° in absence of air) show that after heating for an hour it still retains the greater part of its growth-promoting efficiency.

More is known as to the effect of heat on the anti-scorbutic accessory factor as it occurs in green cabbage leaves [Delf and Tozer, 1918], and in germinating pulses [Delf and Chick, 1919]. In the former case it was shown that fresh green cabbage leaves lose about four-fifths of their anti-scorbutic value when heated in steam for only 20 minutes to a temperature of 100°.

In the experiments to be recorded in this communication, freshly expressed juices of cabbage, swede and orange were contrasted as to their anti-scorbutic values in the raw state and after heating. In all these cases, it was necessary first to find the minimal daily ration of the fresh raw juice which would just afford protection from scurvy when fed to animals on an otherwise scorbutic diet. The technique was that which has been described at length elsewhere [Chick and Hume, 1917; Delf and Tozer, 1918]. Young guinea-pigs were used as the experimental animals and their basal diet was in all cases oats and bran *ad libitum*, and milk previously autoclaved at a pressure of 15 lb., *i.e.* about 120°. The milk ration was given more liberally than hitherto,—to the extent of 60–90 cc. daily, according to the age and appetite of the animal. This increase of the milk ration was justified by control experiments as yet unpublished. The onset of scurvy is not appreciably affected by the increased allowance and healthier animals more resistant to intercurrent disease can be produced, especially in the case of limiting doses which are often difficult to determine on account of secondary infections, as in the case of the orange juice animals which are quoted in Table V.

In Tables I–IV, the data listed in the columns headed "Histology of rib-junctions" were kindly supplied by Miss F. M. Tozer and the diagnoses made as explained by her in a previous communication [Delf and Tozer, 1918]. In our experience, the histology of the rib-junctions may show a subnormal condition (see No. 933, Table IV) or even definite disorganisation of the tissues (as No. 932, Table IV), when the typical clinical symptoms of scurvy in life have been entirely absent. The histological diagnosis then probably indicates a critical condition (which we have called "incipient"), which, if the experiment were sufficiently prolonged, might develop into the typical disease. Similar pre-scorbutic symptoms have been described by McCarrison in connection with the adrenals and the muscular walls of the alimentary tract, both of which suffer marked changes of structure or function when guinea-pigs are kept for short periods on an entirely scorbutic diet, before the usual symptoms of scurvy can be observed [McCarrison, 1919]. He further regards these pre-scorbutic changes as particularly characteristic of a dietary of low vitamine content, and this is in accord with our impression.

Experiments about to be published by Miss F. M. Tozer prove, as we had formerly expected [Delf and Tozer, 1918], that when the anti-scorbutic element is provided in excess, a diet deficient only in the fat-soluble constituent results in a number of changes in the structure of the rib-junctions of guinea-pigs very similar to those observed in the less severe cases of scurvy. In the experiments described below, the amount of milk consumed by the animals satisfies us that the appearances described are primarily due to the lack of sufficient anti-scorbutic in the diet, even when the animal survived the experimental period in apparent health.

Table I. *Experiments with raw green filtered Cabbage Juice.*

No. of animal	Ration of juice cc.	Average amount food consumed daily: Oats & bran g.	Average amount food consumed daily: Milk cc.	Body weight: Initial g.	Body weight: Final g.	Symptoms during life	Duration of experiment, days	Post mortem	Histology of rib-junctions	General result
998	10	19	66	325	638	None	86	Normal	"Incipient" scurvy	Protection
999	10	34	77	345	570	,,	90	,,	,, ,,	,,
999 *C*	5	28	76	325	660	,,	90	,,	Nearly normal	,,
999 *D*	5	32	72	315	580	,,	90	,,	,, ,,	,,
999 *E*	5	47	75	340	617	Tender joints	90	,,	,, ,,	,,
999 *F*	2·5	45	74	340	605	None	86	,,	,, ,,	,,
999 *G*	2·5	33	68	335	375*	,,	74	Haemorrhage on stomach and caecum	Definite scurvy	Died of visceral haemorrhage. (?) Scurvy
999 *H*	2·5	48	67	320	620	,,	87	Normal	Nearly normal	Protection
1105	1·5	57	89	335	745	,,	90	,,	,, ,,	,,
1106	1·5	51	88	335	695	,,	90	,,	,, ,,	,,
1118	0·75	30	63	352	405	Knees swollen, tender; very lame	42	Haemorrhages: fractured knees. Enlarged rib-junctions	Not examined	Typical severe scurvy
1119	0·75	28	72	334	445	,, ,,	42	,, ,,	,, ,,	,, ,,
1130	1·0	40	79	345	510	One knee swollen: abscess	83	Haemorrhage severe in one knee. Tibia fragile	Incipient—Definite	Mild scurvy
1131	1·0	16	61	335	355	Knees swollen, lame	81	Haemorrhage slight. Bones brittle. Ill—pleural adhesion	,, ,,	Scurvy
1132	1·0	24	83	345	675	One knee swollen, teeth loose	81	Teeth loose, otherwise normal	Nearly normal	Protection

* Maximum body weight, 510.

Effect of Heat on the Anti-scorbutic Properties of the Juice of Green Cabbage.

In the case of the cabbage juice, at first only the green outer leaves of fresh cabbages were used. The stouter part of the midrib of each leaf was removed and the remaining part passed through an ordinary mincing machine. The pulp was then wrapped in two thicknesses of stout muslin (mosquito netting) and submitted to a hand-press. The thick green juice was afterwards passed through filter paper, when a clear brownish-green liquid was obtained. Later, by a similar process, the juice was expressed also from the white interior leaves of the cabbages and the freshly prepared green or white juices were fed to different experimental animals.

In the case of the green raw juice, daily rations of 10 cc., 5 cc., 2·5 cc. and 1·5 cc. were all found to give perfect protection from symptoms of scurvy during the experimental period (60–90 days, Table I). It was remarkable that the strongest and best grown animals were on the smallest of these doses (1·5 cc., Fig. 1 *E*), but these two had a large appetite for autoclaved milk (Table I). When this dose was halved and fed to another set of animals, typical and severe scurvy developed after a brief period of normal growth (35–45 days, Fig. 1 *F*). Three animals were subsequently given a ration of 1 cc. fresh juice. Two of these showed slight signs of scurvy at 80 days; the third was normal, except for loose teeth. The minimum raw protective dose is therefore just above 1 cc. daily. Parallel experiments with juice from the white leaves lead to the conclusion that the anti-scorbutic value of the two kinds of juices is not very different, 1·5 cc. affording apparent protection from scurvy.

The juice prepared as described above was placed in small flasks stoppered with cotton-wool plugs and heated in steam at 100° for an hour, or for twenty minutes in the different experiments. A soft whitish coagulum was produced by the heating and this was included in the doses fed to the animals.

Successive experiments with the juice heated for one hour showed that on a daily dose of 5 cc. or less all the animals developed severe scurvy (Table II *a*). Of two animals on a 7·5 cc. dose one remained in perfect health for 90 days when the experiment was terminated, and one developed severe scurvy after 20 days. The latter animal survived in fair condition for 90 days, but was badly crippled in the hind-quarters which at the post mortem showed typical scurvy lesions. Two others on a 10 cc. ration were protected. Evidently a ration of 7·5 cc. is near the limit required for complete protection and perhaps may be regarded as the equivalent of 1 cc. of the raw juice. This indicates a loss of about 80 % of the original value during the hour of heating.

Experiments have also been made in which animals were fed on the juice heated for 20 minutes. Two animals on a 5 cc. dose early developed symptoms of scurvy—No. 1127 on the 26th and No. 1128 on the 28th day. Three other

Table II. *Experiments with green filtered Cabbage Juice heated to* 100°.

(*a*) Heated for 1 hour.

No. of animal	Ration of juice cc.	Average amount food consumed daily: Oats & bran g.	Milk cc.	Body weight: Initial g.	Final g.	Symptoms during life	Duration of experiment, days	Post mortem	Histology of rib-junctions	General result
1107	3·0	29	64	325	400	Both knees swollen, sore; lame	29	Haemorrhages extensive: fractured knees; enlarged rib-junctions	Scurvy	Severe scurvy
1108	3·0	32	82	330	540	,, ,,	32	,, ,,	Severe scurvy	,, ,,
1100	5	19	69	336	463	,, ,,	90	Haemorrhages extensive: loose teeth	Definite ,,	Scurvy; some protection
1101	5	40	79	325	525	Left knee swollen, sore, lame	91	,, ,,	Incipient ,,	,, ,,
1102	5	31	76	330	527	Both knees swollen, sore, lame	89	Haemorrhages extensive: loose teeth, rib-junctions enlarged	Incipient—definite scurvy	,, ,,
1122	5	19	55	335	366	,, ,,	35	Haemorrhages severe, knees fractured	Severe scurvy	Severe scurvy
1120	7·5	25	83	335	550	No symptoms	90	Normal. 3 rib junctions enlarged	Definite scurvy	Scurvy
1121	7·5	20	79	340	380	Knees swollen: crippled	65	Severe haemorrhages: fractured knees, enlarged rib-junctions	Definite—severe	Severe scurvy
1139	10	20	83	350	615	Slight soreness, improved	60	Still alive	—	Alive, doing well
1140	10	27	83	320	540	Lame, probable injury	60	Abscess in lung	Not examined	Protected

(*b*) Heated for 20 minutes.

1127	5	18	75	335	340	Right knee swollen, scurvy position	62	Severe haemorrhages: fractured knees, enlarged rib-junctions	Not examined	Severe scurvy
1128	5	20	77	328	465	Right knee swollen, lame	89	Severe haemorrhages, bones fragile	Incipient—Definite	Scurvy
1129	7·5	16	83	335	475	Both knees swollen	83	Haemorrhages slight—rib junctions enlarged	Severe scurvy	,,
1137	7·5	15	58	342	385	One knee swollen	54	Haemorrhages, fragile tibia, teeth loose	Incipient	,,
1138	7·5	25	69	340	480	,, ,, ,,	46	,, ,, ,,	—	,,

animals received a ration of 7·5 cc. daily; one of these became scorbutic after 28 days; the other two were killed at 54 and 46 days respectively and both showed distinct signs of scurvy.

These results fall into line with those previously obtained for the green leaves [Delf and Tozer, 1918], in indicating a considerable destruction of the anti-scorbutic value after heating, and the destruction is, if anything, greater in the expressed juice than in the leaf tissue. The experiments with the juice heated for only 20 minutes show how great an amount of destruction of anti-scorbutic substance occurs at the beginning of heating, the loss after an hour being indistinguishable from that after twenty minutes' heating.

The Effect of Heat on the Anti-scorbutic Properties of the Juice of the Turnip-swede (*Brassica campestris* var. *Napo-brassica*).

An account has already been published of results of experiments made by Chick and Rhodes [1918], working in this laboratory, which establish that 2·5 cc. may be regarded as the minimum daily protective ration of raw swede juice. In the winter of 1918–1919, these results were confirmed and extended to the heated juices as summarised in Tables III and IV. I am indebted to Miss D. Gardiner of Girton College for much assistance in the preparation of the juices and in the care of the experimental animals.

The juice was prepared daily by the method (previously adopted by Chick and Rhodes) of rubbing a fresh surface of the root on an ordinary kitchen grater, and squeezing the pulp thus obtained by hand through coarse muslin. Out of four animals given 2·5 cc. daily as the sole anti-scorbutic in their diet, two remained in perfect health throughout the experiment, another was slightly scorbutic but was otherwise in fairly good condition (No. 953, Table III), and the fourth died after 36 days of some intercurrent disease, with accompanying symptoms of mild scurvy. On smaller doses severe scurvy always appeared and on a larger dose protection was achieved in every case.

The fresh swede juice was heated for an hour either in test-tubes plugged with cotton-wool and nearly submerged in a water-bath kept at 80°, or in small flasks also plugged with cotton-wool but heated in a steam chamber at 100°, or in an autoclave at about 10 lb. pressure, *i.e.* about 110°, or at 25 lb. pressure, *i.e.* about 130°. Perfect protection from scurvy was given by a 5 cc. dose of the juice previously heated at either 80° or 100° or even 110°. This dose failed to protect from scurvy if previously heated for an hour at 130° (Table IV), but when the dose was increased to 10 cc., protection was achieved in the case of two experimental animals (Table IV).

Swede juice is thus much more stable at high temperatures than is cabbage juice, double the amount of the raw minimal dose giving protection after heating for an hour at 100° or 110°, whereas with raw cabbage juice about seven times the raw minimal dose is necessary to give this protection after heating for an hour at 100°.

Table III. *Experiments with raw Swede Juice.*

No. of animal	Average amount food consumed daily: Ration swede juice cc.	Oats & bran g.	Milk cc.	Body weight: Initial g.	Final g.	Symptoms during life	Duration of experiment, days	Post mortem	Histology of rib-junctions	General result
938	5	21	69	325	543	None	88	Teeth loose, brittle	Normal	Protection
939	5	23	53	330	387	,,	51	Tibia somewhat brittle	Nearly normal	,,
968	5	21	74	350	487	,,	91	Teeth brittle: rib-junctions somewhat enlarged	,, ,,	,,
971	5	24	60	335	512	,,	91	Rib-junctions slightly enlarged	,, ,,	,,
972	5	52	75	345	617	,,	91	Teeth rather brittle	,, ,,	,,
952	2·5	18	55	330	325	Joints swollen: infection	36	Muscular and subcutaneous haemorrhages—slight	Incipient scurvy	Infection, mild scurvy
953	2·5	17	59	330	375	Limbs tender when examined	90	Slight subcutaneous haemorrhages	Scurvy	Protection incomplete, mild scurvy
970	2·5	19	75	325	530	,, ,,	90	Normal	Normal	Protection
973	2·5	30	75	340	550	None	91	,,	Nearly normal	,,
955	1·5	31	70	320	505	,,	90	Brittle teeth	,, ,,	,,
956	1·5	16	58	320	350	,,	45	Haemorrhage on gut	,, ,,	,,
962	1·5	12	70	320	388	Scurvy position, knees swollen	90	Subcutaneous haemorrhages; fractured knees, etc.	Scurvy	Scurvy
978	1·5	15	56	320	340	Limbs very tender on handling	71	Muscular haemorrhages; rib-junctions enlarged	,,	,,
979	1·5	21	54	325	395	Lame, body very thin	71	Muscular haemorrhages; rib-junctions enlarged; knees fractured	,,	,,
980	0·75	15	58	320	315	Lame; swollen knees	42	Severe haemorrhage, etc.	,,	Severe scurvy
981	0·75	16	59	320	320	,, ,,	41	,, ,,	,,	,, ,,
982	0·75	14	51	325	290	,, ,,	34	,, ,,	,,	,, ,,

Effect of Heat on the Anti-scorbutic Value of Fresh Orange Juice.

I am indebted to Miss E. M. Hume and Miss A. J. Davey for permission to publish the data quoted in Table V, from which may be seen the results of their experiments with raw orange juice. From the experiments of Miss Hume carried out in 1917, it will be seen that 3 cc. of the juice affords ample protection from scurvy in young guinea-pigs and that fairly good growth was made when autoclaved milk was included in the dietary. The results of a dose of 1·5 cc. were variable, only five out of eleven animals receiving adequate protection from scurvy (Table V, Nos. 720, 721, 723, 744, and 1000). Of these only No. 1000, which had the largest appetite for milk, made a practically normal growth curve (Fig. 4 *B*). Two of the animals (Nos. 743 and 1001) were presumably in a "pre-scorbutic" condition, showing histological deformities without any clinical symptoms of scurvy. On the whole, we may conclude from these experiments that 1·5 cc. is the minimal protective ration of the freshly squeezed juice from oranges in good condition when fed to animals which can be kept free from other forms of disease.

Freshly squeezed orange juice was heated for an hour at three different temperatures. At 70° in a water-bath as for the swede juice; at higher temperatures in a steam chamber or autoclave as already described for the other juices.

One case of protection was observed on a 1·5 cc. dose, heated at 70°, but this was probably an exceptional animal since a larger ration given later in the year failed to give complete protection (3 cc., Table VI). Animals were kept successfully in good health throughout the experimental period both on 3 cc. rations and on 1·5 cc. rations heated at 100°; but in the former case two out of four animals were found to exhibit post mortem and histological signs of scurvy (perhaps attributable to the difficulty of getting good oranges at that time), whilst in the latter case two out of three animals showed similar signs of a scorbutic or rather pre-scorbutic condition (weight charts, Fig. 4 *C*, *D*). Two animals on a 3 cc. dose heated at 130° were protected from scurvy and a third nearly protected, but three other animals on a 2 cc. ration of this juice developed severe scurvy. Orange juice is therefore still more stable than swede juice, less than double the raw minimal dose affording protection after an hour of heating at 130°.

This stability of the orange juice to heat suggested the possibility that the acidity present in the juice might have a stabilising effect on the anti-scorbutic substance, and led to experiments carried out mainly by Miss B. F. Runge in which the juice was nearly neutralised with 10 % sodium carbonate prior to heating. The alkaline solution was added drop by drop until the liquid was just acid to a standard solution of dibromo-*o*-cresolsulphonephthalein (P_H 5·2–6·8). The residual acidity of the juice was then about the same as the slight natural acidity of the swede and cabbage juices used in the other experiments.

Table IV. *Experiments with Swede Juice heated for* 1 *hour.*

No. of animal	Ration swede juice cc.	Temp. of heating °C.	Average amount food consumed daily: Oats & bran g.	Average amount food consumed daily: Milk cc.	Body weight: Initial g.	Body weight: Final g.	Symptoms during life	Duration of experiment, days	Post mortem	Histology of rib-junctions	General result
931	5	80	22	67	337	410	No symptoms	84	Haemorrhage on small intestine	Incipient scurvy	Protection, possible infection
932	5	80	21	71	355	465	,, ,,	82	Normal	Scurvy	,, ,,
940	5	80	13	66	322	390	Lung trouble; scurvy also	92	Subcutaneous and muscular haemorrhages; bones fragile	,,	Partial protection
933	5	100	18	69	320	480	No symptoms	90	Teeth brittle	Incipient scurvy	Protection
934	5	100	20	72	340	517	,, ,,	92	,, ,,	,, ,,	,,
941 *A*	5	100	26	68	330	412	,, ,,	90	One rib-junction much enlarged	,, ,,	,,
943	5	110	10	60	330	370	Limbs tender after 38th day	85	Lungs inflamed; intestinal ulcers	Nearly normal	,,
944	5	110	12	75	335	485	,, ,, 34th ,,	91	Teeth brittle	Incipient scurvy	,,
945	5	110	16	68	322	485	,, ,, 22nd ,,	92	Rib-junctions ridged	,, ,,	,,
936	10	130	18	67	350	498	,, ,, 29th ,,	88	Slight muscular haemorrhages; intestinal ulcers	,, ,,	,,
937	10	130	30	74	310	540	,, ,, 34th ,,	90	Teeth brittle	Normal	,,
974	5	130	13	53	341	320	Scurvy position, lame after 21st day	33	Muscular haemorrhages, fragile bones, etc.	Not examined	Typical acute scurvy
975	5	130	15	58	340	335	,, ,, 20th ,,	34	,, ,,	,, ,,	,, ,,
976	5	130	17	58	320	287	,, ,, 15th ,,	31	,, ,,	,, ,,	,, ,,

Three animals given 3 cc. of this juice heated for an hour at 100° and three others given only 1·5 cc. daily were successfully protected from scurvy during the experimental period, the condition of those on the larger ration being rather better than of those on the smaller. Neutralising nearly all the excess of acid in orange juice therefore does not appreciably reduce the stability of the anti-scorbutic constituent of the juice at a temperature of 100°.

Experiments were also made with the juice of oranges which had been canned and stored at laboratory temperatures for five months. The canning process involved heating in closed cans for 20–30 minutes, the temperature gradually rising from about 80° to 100° and remaining at 100° for not more than five minutes. A certain amount of water was added to the fruit after packing the cans and this was included in the juice afterwards expressed by hand from the fruit when the cans were opened. Allowing for the added water, a dose equivalent as nearly as possible to 1·5 cc. of the fresh juice was given daily to three experimental animals and afforded them adequate protection from scurvy for 88 days, when the experiment was terminated. Canned oranges produce a bitter juice and are probably of no commercial value, but this result with fruit in which the value of the raw juice is well known indicates the possibilities of investigations with other fruit juices.

Discussion of Results.

Experiments have been described in which the juice of fresh cabbage, swede and orange has been fed to animals as the sole anti-scorbutic element in a basal diet of oats and bran *ad libitum*, and 60–90 cc. autoclaved milk daily. From these experiments it can be affirmed that under these conditions the minimal daily dose of the raw juice for the adequate protection from scurvy of young guinea-pigs is about 1·0 cc. cabbage, 2·5 cc. swede, and 1·5 cc. orange juice respectively.

When these juices are subjected to temperatures up to 130° a corresponding reduction of their anti-scorbutic properties appears. This reduction is greatest in the case of cabbage juice and is least in the case of orange juice. After being heated at 100° for an hour, at least $7\frac{1}{2}$ times the raw dose in the case of cabbage, or twice the raw dose in the case of swede, is necessary to give protection from scurvy, whereas orange juice does not appear to have deteriorated appreciably during this period of heating. Only at 130° was any definite loss of value detected in the orange juice; 2 cc. was then an insufficient dose for protection, but 3 cc. gave the necessary protection after heating at this temperature.

In considering these results, it is interesting to find that orange juice, which is the most stable of the juices tested, is also the most acid in reaction. As long ago as 1912, Holst noticed that fruit juices were more heat-stable than vegetable juices, and he suggested that the stability was due to the presence of acid [Holst and Frölich, 1912]. That the hydrogen ion concentration may have an important influence on the stability of this vitamine is

Table V. *Experiments with raw Orange Juice: basal diet oats, bran and autoclaved milk.*

No. of animal	Date of starting experiment	Ration orange juice cc.	Average amount food consumed daily: Oats & bran g.	Milk cc.	Body weight: Initial g.	Final g.	Symptoms during life	Duration of experiment, days	Post mortem	Histology of rib-junctions	General result
79	21.i.17	5	35	60	290	420	Limbs tender	106	Normal	Incipient scurvy	Protection
111	25.i.17	5	30	74	315	520	" "	89	Rib-junctions slightly ridged	Normal	"
239	1.v.17	3	52	55	365	598	None	93	Normal	"	"
240	3.v.17	3	65	64	300	365	"	74	Practically normal	Nearly normal	"
1013	13.i.19	3	52	76	336	605	Knees swollen, tender after 53rd day	91	Normal	Normal	"
1014	25.i.19	3	41	60	342	514	None	91	"	"	"
720	9.iii.18	1·5	63	57	342	Max. (490) 378	"	62	Intestine probably infected; spots on liver	Nearly normal	"
721	11.iii.18	1·5	85	60	335	510	"	90	Normal	Incipient scurvy	"
722	13.iii.18	1·5	66	54	360	372	"	46	Haemorrhages in stomach and caecum; two rib-junctions enlarged	Severe scurvy	Scurvy
723	13.iii.18	1·5	39	51	336	(473) 245	"	68	Tibia firm, femur brittle; digestive tract unhealthy	Definite scurvy	Protection
742	11.v.18	1·5	25	53	340	260	Swollen knees; crippled	50	Fragile bones; intramuscular haemorrhages on knees	Severe scurvy	Scurvy
743	17.v.18	1·5	43	59	341	318	Emaciation after 70 days	83	Bones slightly fragile	Definite scurvy	"
744	6.vi.18	1·5	49	58	337	(472) 368	No symptoms	62	Teeth brittle	Incipient scurvy	Protection—died of unknown illness
1000	6.i.19	1·5	56	78	337	651	" "	90	Normal	Nearly normal	Protection
1001	9.i.19	1·5	43	58	334	287	" "	73	Tibiae fragile; rib-junctions ridged, one heavily	Severe scurvy	Partial protection
1002	11.i.19	1·5	36	62	339	340	Pneumonia	65	Liquid in pleural cavity; haemorrhages on caecum	Definite scurvy	Partial protection; pneumonia
1003	11.i.19	1·5	24	53	338	263	No symptoms	88	Dark inflammatory patches on lungs	" "	Partial protection; lung trouble
745	14.vi.18	0·5	37	58	340	(474) 400	Joints swollen, tender, scurvy position	59	Haemorrhages in muscles of knees; rib-junctions slightly enlarged	Incipient scurvy	Slight scurvy
1025	3.iii.19	0·5	22	64	339	267	Swollen knees on 22nd day, scurvy position	75	" "	Severe scurvy	Severe scurvy
1026	4.iii.19	0·5	22	63	331	360	Swollen knees after 38 days, scurvy position	91	" "	Definite scurvy	Scurvy
1027	6.iii.19	0·5	30	56	331	309	Knees swollen after 34 days	58	" "	" "	"
1028	8.iii.19	0·5	24	59	330	464	Knees swollen after 43 days	89	Haemorrhages slight; bones slightly fragile	" "	"
1029	11.iii.19	0·5	40	59	340	311	Knees swollen after 29 days	43	Bones slightly fragile; rib-junctions much swollen	Severe scurvy	Severe scurvy
1030	17.iii.19	0·5	18	59	343	230	Knees swollen after 20 days	66	Haemorrhages; bones fragile; rib-junctions swollen	" "	" "

Table VI. *Experiments with heated Orange Juice: basal diet oats, bran and autoclaved milk.*

A. Heated orange juice.

No. of animal	Date of starting experiment	Ration of orange juice cc.	Temp. of heating °C.	Average amount food consumed daily: Oats & bran g.	Milk cc.	Body weight: Initial g.	Final g.	Symptoms during life	Duration of experiment, days	Post mortem	Histology of rib-junctions	General result
871	10. vi. 18	3	70	37	59	330	550	Joints slightly tender	90	Slight muscular haemorrhage; fragile bones	Severe scurvy	Scurvy, but good health
857	23. iv. 18	1·5	70	37	57	331	652	,, ,, ,,	94	Slight haemorrhage on intestine	Nearly normal	Protection
858	23. iv. 18	1·5	70	29	47	326	318	,, ,, ,,	26	,, ,,	,, ,,	Protection; death from unknown cause
905	7. ix. 18	3	100	36	60	350	593	,, ,, ,,	90	Teeth loose; tibiae brittle	Incipient scurvy	Protection
906	7. ix. 18	3	100	18	60	345	500	,, ,, ,,	90	Rib-junctions swollen: slight muscular haemorrhage	Definite scurvy	Partial protection; slight scurvy
949	1. xii. 18	3	100	22	68	320	630	No symptoms	90	Normal	Normal	Protection
950	2. i. 19	3	100	24	70	325	598	,, ,,	89	,,	,,	,,
986	25. iv. 19	1·5	100	22	75	320	610	,, ,,	92	One rib-junction enlarged; the rest normal	Enlarged junction fractured; the rest normal	Protection; trace scurvy (?)
987	21. iv. 19	1·5	100	14	63	328	485	,, ,,	94	Slight muscular haemorrhage	,, ,,	,, ,,
988	25. iv. 19	1·5	100	22	74	320	670	,, ,,	89	Rib-junctions slightly enlarged	Nearly normal	
951	4. i. 19	3	130	10	60	325	335	Knees tender, a little lame	72	Emaciated; lungs inflamed	Definite scurvy	Protection incomplete
959	13. i. 19	3	130	17	63	320	525	Knees tender	90	Rib-junctions slightly ridged	Normal	Protection
960	19. i. 19	3	130	15	66	340	402	,, ,,	90	,, ,,	Incipient scurvy	,,
983	8. iv. 19	2	130	23	60	325	350	Knees much swollen: very painful	40	Severe haemorrhages; fragile bones	Severe scurvy	Severe scurvy
984	8. iv. 19	2	130	16	69	330	430	,, ,, ,,	78	,, ,,	,, ,,	,, ,,
985 *A*	9. iv. 19	2	130	15	72	320	450	,, ,, ,,	85	,, ,,	,, ,,	,, ,,

B. Heated nearly neutralised orange juice.

989	17. v. 19	3	100	17	74	325	535	No symptoms	92	No symptoms	Incipient scurvy	Protection
990	17. v. 19	3	100	23	74	325	670	,, ,,	92	Fatty and subcutaneous tissue injected	Normal	,,
991	21. v. 19	3	100	27	76	322	600	,, ,,	90	Normal	Incipient scurvy	,,
992	27. v. 19	1·5	100	21	72	323	515	,, ,,	89	Jaw and lower teeth somewhat brittle	,, ,,	,,
993	27. v. 19	1·5	100	20	72	325	585	,, ,,	90	Rib-junctions all slightly enlarged	,, ,,	,,
994	27. v. 19	1·5	100	20	74	330	510	,, ,,	89	Normal	,, ,,	,,

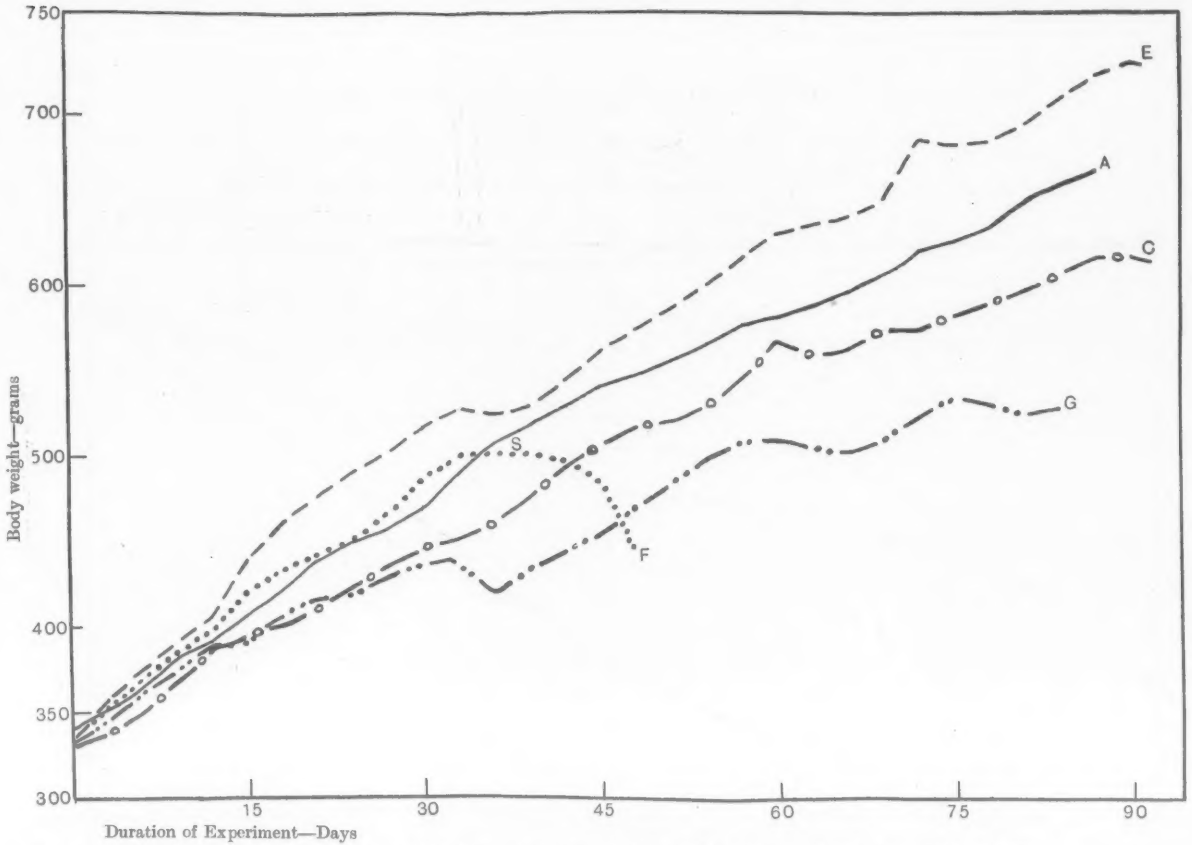


Fig. 1. Weight charts showing growth of animals fed on oats, bran, autoclaved milk and raw or heated green cabbage juice.

A = Mean weight chart of four animals: showing standard growth on normal diet of oats, bran and 30 g. fresh cabbage daily and water.
C = „ „ „ Nos. 999 *C*, *D*, *E*, on 5 cc. raw filtered green cabbage juice.
E = „ „ „ Nos. 1105, 1106 on 1·5 cc. „ „ „
F = Weight chart of No. 1119 on 0·75 cc. raw filtered green cabbage juice. At *S* symptoms of scurvy first appeared.
G = „ „ No. 1120 on 7·5 cc. green filtered cabbage juice heated for an hour at 100° C.

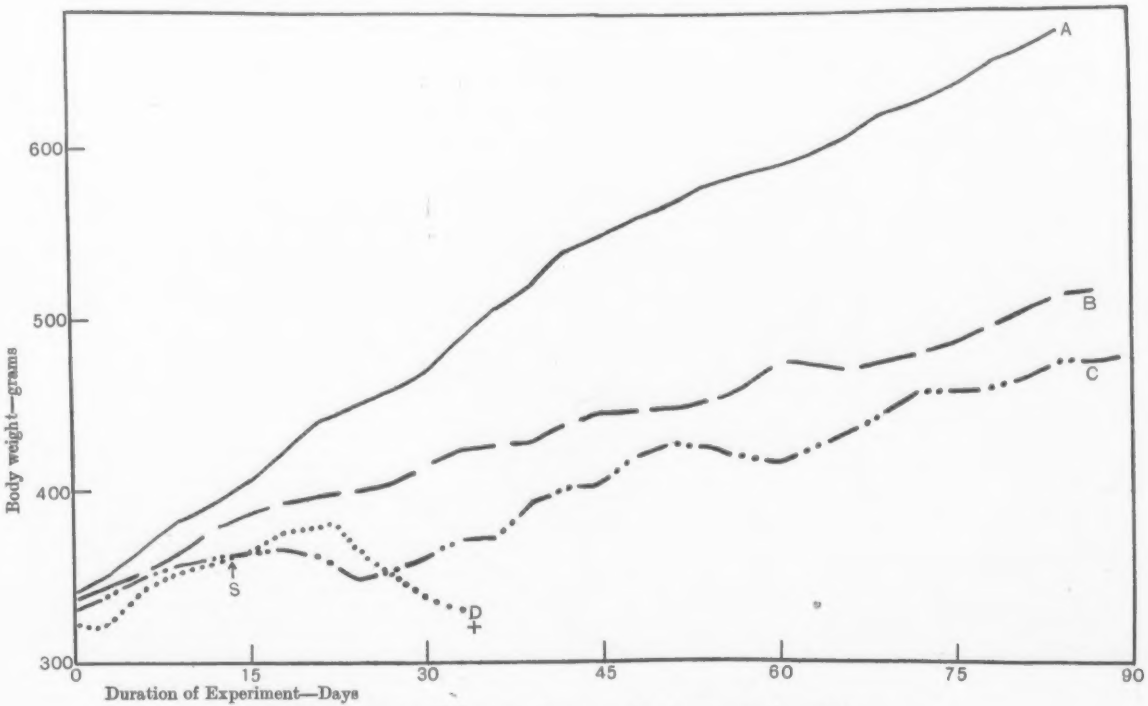


Fig. 2. Weight charts of animals on oats, bran, autoclaved milk and raw swede juice.

A = Standard weight chart on diet of oats, bran, water and 30 g. fresh cabbage daily.
B = Mean weight chart of Nos. 938, 968, 971, 972, on a ration of 5 cc. raw swede juice.
C = " " Nos. 953, 970, 973, " " 2·5 cc. " "
D = " " Nos. 980, 981, 982, " " 0·75 cc. " "
At *S* symptoms of scurvy first appeared; at + No. 982 died of scurvy.

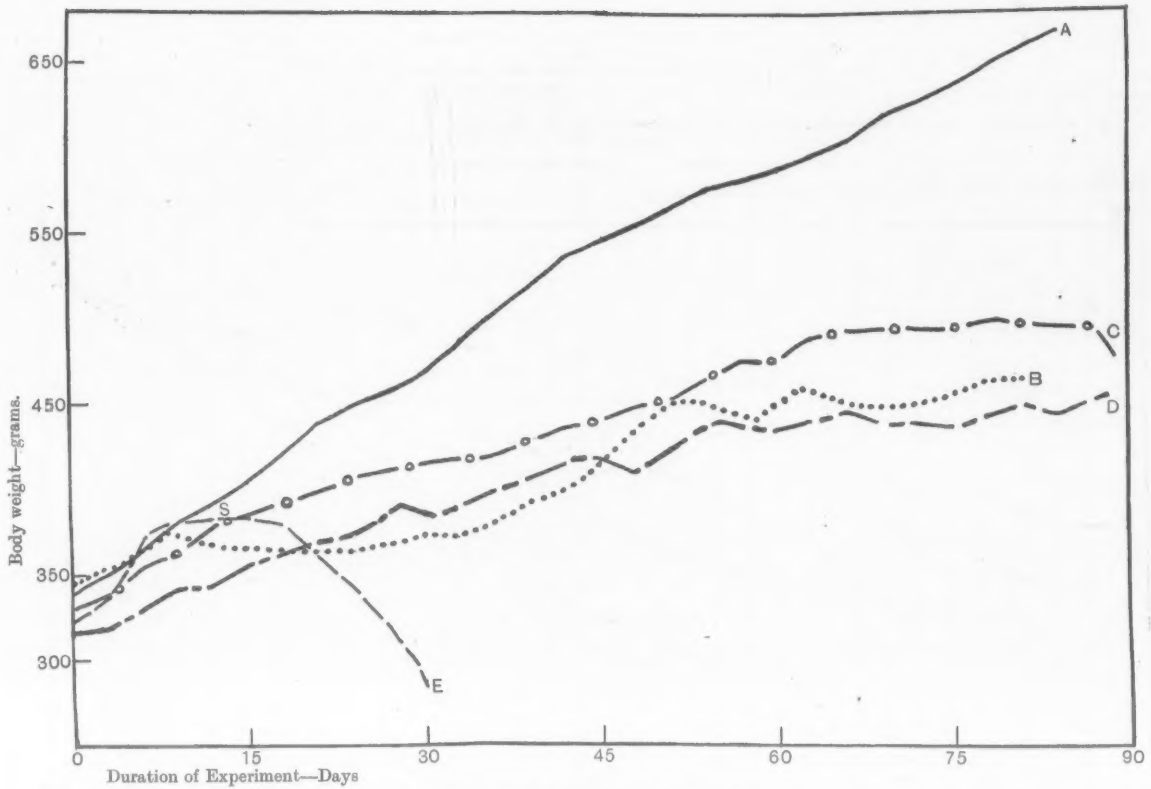


Fig. 3. Weight charts of animals fed on oats, bran, autoclaved milk and heated swede juice.

A = Standard weight chart of normal growth (as for Figs. 1, 2).
B = Weight chart of No. 932 on 5 cc. swede juice heated for 1 hour at 80° C.
C = Mean weight chart of Nos. 933, 934, 941 *A*, on 5 cc. swede juice heated for 1 hour at 100° C.
D = „ „ Nos. 937, 961, on 10 cc. swede juice heated for 1 hour at 130° C.
E = Weight chart of No. 977, on 5 cc. swede juice heated for 1 hour at 130° C. At *S* symptoms of scurvy first appeared.

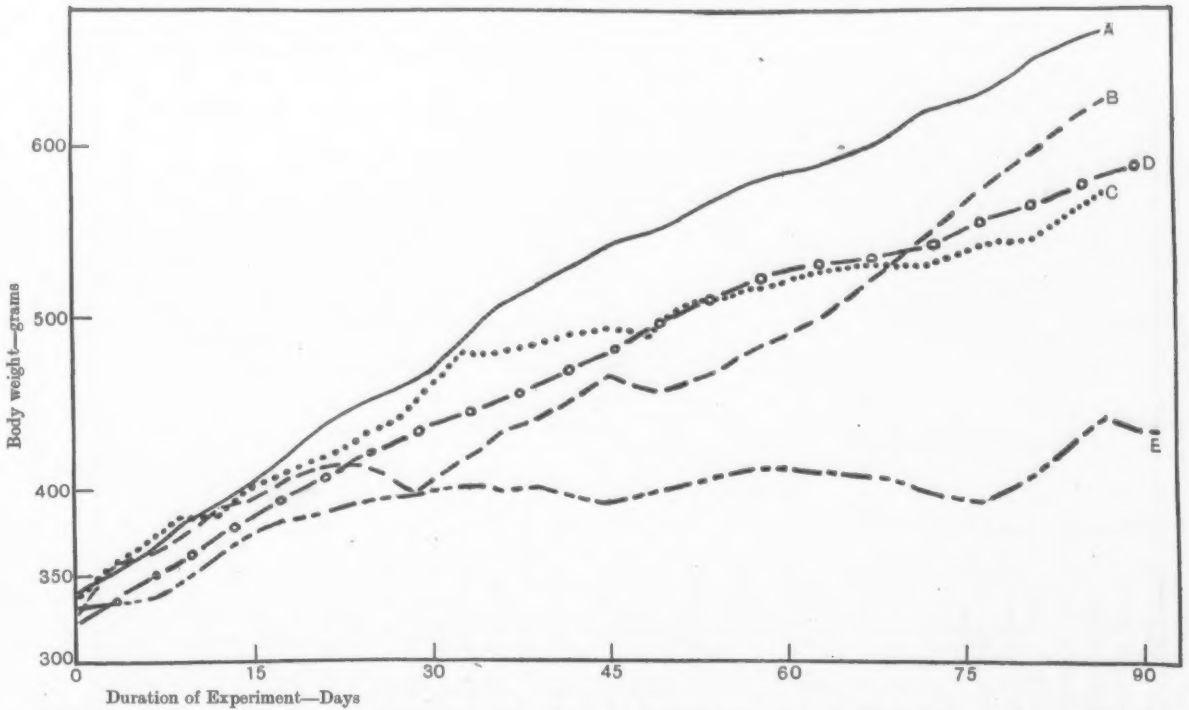


Fig. 4. Weight charts showing growth of animals on a diet of oats, bran, autoclaved milk and a ration of raw or heated orange juice daily.
A = Standard curve of normal growth (as in Figs. 1–3).
B = Weight chart of No. 1000, on 1·5 cc. raw orange juice (Table V).
C = Mean weight chart of Nos. 949, 950, 905, 906, on 3 cc. fresh orange juice heated to 100° C. for an hour.
D = " " Nos. 986, 987, 988, on 1·5 cc. fresh orange juice heated to 100° C. for 1 hour.
E = " " Nos. 959, 960, on 3 cc. fresh orange juice heated to 130° C. for 1 hour.

shown by the experiments of Harden and Zilva [1918], who found that orange juice which was made only slightly alkaline lost almost immediately its anti-scorbutic value. In the experiments described above, the raw juices of cabbage and swede have about the same hydrogen ion concentration, but this slight acidity is accompanied by very different degrees of stability in the two cases. The greater stability of the orange juice to heat was maintained even when the juice was nearly neutralised before heating.

A further point of difference between the three juices tested may be found in their effect on the growth of the experimental animals. A glance at Figs. 1–4 (pp. 223–6) will give the general impression that the cabbage juice animals were on the whole the most satisfactory in this respect, and the variability in the consumption of milk does not altogether account for this[1]. It is well known that green vegetables have growth-promoting as well as anti-scorbutic properties and other workers in this Institute have obtained evidence (as yet unpublished) that this growth-promoting power is also shared to some extent by the expressed juice. No appreciable growth is obtained, however, when guinea-pigs are fed on either cabbage, swede or orange juices in doses up to 10 cc. if there is no other source of fat-soluble substance in the diet. In the above experiments therefore, the apparent limitation of growth in the case of certain groups of animals in spite of their considerable allowance of milk suggests that a low ration of anti-scorbutic in a diet may also limit the availability of the fat-soluble substance to the animal, even when protection from scurvy has been secured. In the case of the animals to which swede juice was fed (Table III), if we except No. 939 (on 5 cc.) and No. 952 (on 2·5 cc.), both of which died early in the course of the experiment with some unknown complaint, the amount of milk consumed by the two lots is about the same, but the average rates of growth of the animals are different (Fig. 2 *B*, *C*); whilst in both these cases the growth of none of the animals approached that of the normal growth on a cabbage diet (Fig. 2 *A*). In the case of animals on 3 cc. heated orange juice (Table VI), the consumption of milk was nearly equal in the cases of the two groups of animals, but those on the juice heated to 130° made very little growth although the ration of juice given proved to be just above the scurvy limit (Fig. 4 *C*, *E*). Growth seems therefore to be affected by the limitation of the anti-scorbutic element in the diet apart from the appearance of definite symptoms of scurvy and apart from deficiency in the growth-promoting vitamines.

With regard to the somewhat unexpected stability of swede and orange juice at temperatures above 100°, this is parallel with what was previously found with green cabbage leaves. It must be remembered that the heating at these temperatures was done in a closed autoclave in the absence of air. This may well affect the rate of destruction either directly by retarding oxidation or indirectly by the production of stabilising bodies. The rather

[1] Cp. Nos. 970, 973, Table III with 999 *F*, *H*, Table I, both on 2·5 cc. doses; or Nos. 938, 968, 972, Table III with 999 *C*, *D*, *E*, Table I, both on 5 cc. doses.

surprising stability at 130° in the absence of air suggests that there may be advantage in adopting methods of canning fruit or vegetables at temperatures above boiling-point for as short a time as possible to ensure sterility. Further investigations into the value of canned products would appear to be desirable.

In conclusion I have to thank Dr Harriette Chick for suggestions and advice especially during the earlier stages of the investigation, and Miss F. M. Tozer for permission to quote the results of her histological investigations of the rib-junctions of the animals used in these experiments.

REFERENCES.

Chick and Hume (1917). *J. Soc. Trop. Med. Hyg.* **10**, 141.
Chick and Rhodes (1918). *Lancet*, ii, 774.
Delf and Chick (1919). *Biochem. J.* **13**, 201.
Delf and Tozer (1918). *Biochem. J.* **12**, 416.
Drummond, J. (1919). *Biochem. J.* **13**, 81.
Halliburton and Others (1919). *J. Physiol.* **52**, 328.
Harden and Zilva (1918). *Lancet*, ii, 320.
Holst and Frölich (1912). *J. Hyg.* **72**, 1.
McCarrison (1919). *Indian J. Med. Research*, **7**, 188.
McCollum and Simmonds (1918). *J. Biol. Chem.* **33**, 55.
Steenbock, Boutwell and Kent (1918). *J. Biol. Chem.* **25**, 517

XXIV. THE PRODUCTS OF THE "ACETONE: *n*-BUTYL ALCOHOL" FERMENTATION OF CARBOHYDRATE MATERIAL WITH SPECIAL REFERENCE TO SOME OF THE INTERMEDIATE SUBSTANCES PRODUCED.

By JOSEPH REILLY, WILFRED JOHN HICKINBOTTOM, FRANCIS ROBERT HENLEY, and AAGE CHRISTIAN THAYSEN.

From the Royal Naval Cordite Factory, Holton Heath.

(*Received March 5th, 1920.*)

The production of butyl alcohol and other higher alcohols from carbohydrate material, directly by fermentation, has been well-established [compare Fitz, 1878, 1882, 1883, 1884; Gruber, 1887; Perdrix, 1891; Grimbert, 1893; Botkin, 1893; Schardinger, 1907; Bredemann, 1909; Buchner and Meisenheimer, 1908].

The presence of iodoform-producing substances among the products of fermentation has been recorded occasionally, but the substance yielding the iodoform has never been identified. The first observation of acetone as the result of fermentation of carbohydrates was made by Schardinger [1905], who obtained acetone together with acetic and formic acids. The isolation of an organism yielding acetone and *n*-butyl alcohol from amylaceous material was due to Fernbach and Strange [1912]. Since this initial discovery other processes have been described which produce acetone and *n*-butyl or ethyl alcohol by the breakdown of carbohydrates under bacterial action [Farbenfabr. vorm F. Bayer & Co.; Weizmann, 1915, 1919; Ricard, 1918; Northrop, 1919, 1 & 2]. While the technique of fermentations yielding acetone and *n*-butyl alcohol has improved so that it is possible to carry them out successfully on a large scale, no investigation on the mechanism of the process appears to have been recorded.

A quantitative examination was therefore undertaken of certain intermediate substances formed. The normal end-products of the fermentation, as estimated during the course of the present work, are shown in the following Table I. The total weight of end-products exceeds the weight of starch fermented, but the total carbon content is nearly equivalent to that of the starch. Hence it may be inferred that the starch undergoes hydrolysis.

Table I.

From Observations made at the Royal Naval Cordite Factory, Holton Heath.

1000 lb. maize containing 650 lb. starch. Volume of mash (6·5 % maize) = 1540 gallons.

		lb.	Carbon content
650 lb. starch yield		70 acetone	43·5
	3410 cu. ft. of CO_2 evolved—measured at 27° and 760 mm.	163 *n*-butyl alcohol = 390 CO_2 evolved	105·7 106·3
1 vol. of mash dissolves 0·555 vol. CO_2 at 38°	135 cu. ft CO_2 in solution at 0° and 760 mm.	= 17 CO_2 in solution	4·6
	2090 cu. ft. H_2 at 27° and 760 mm.	= 11 H_2	—
Total gas evolved (Composition of gas assumed to be constant throughout the fermentation)	= 5·5 cu. ft. at 27° and 760 mm. of mixed gas per 1 lb. of maize fermented		
	Residual acidity*	= 12·0 containing	5·7
		663·0	265·8

Theoretically 650 lb. of starch is equivalent to 722 lb. of hexose. The carbon content of these amounts to 288·8 lb.

* The residual acidity is taken as acetic acid 56·5 % by weight, and butyric acid 43·5 % by weight. The non-volatile portion is recorded as butyric acid for the purpose of calculation. (See Table III, p. 232, and pp. 246–7.)

Calculated as Percentage of Starch Fermented.

100 g. starch gives 111·1 g. hexose and contains 44·4 g. carbon

	g.	Carbon
100 g. starch gives	10·77 acetone	6·68 g.
„ „	25·07 *n*-butyl alcohol	16·21
„ „	62·61 carbon dioxide	17·07
„ „	1·60 hydrogen	—
„ „	1·80 residual acidity	0·85
	101·85	40·81

In a normal fermentation several remarkable features may be observed. The acidity of the mash increases from a very small initial value until a maximum is reached in from 13–17 hours after inoculation. (The length of time taken to reach this point is influenced by the percentage of inoculant used, temperature of the mash, etc.) When the maximum acidity is reached in 6·5 % maize mash, 3·5–4·5 cc. of $N/10$ alkali are required to neutralise 10 cc. of mash. After this point is reached a very marked acceleration in the rate of production of acetone, *n*-butyl alcohol, carbon dioxide and hydrogen takes place. The acidity gradually falls to a constant value, 1·5–2·5 cc. of $N/10$ alkali being required to neutralise 10 cc. of mash. (See Fig. 1, Curve 2.)

The rate of gas evolution is shown in Curve 1, Fig. 1, and the composition of the gas in Table II, p. 232.

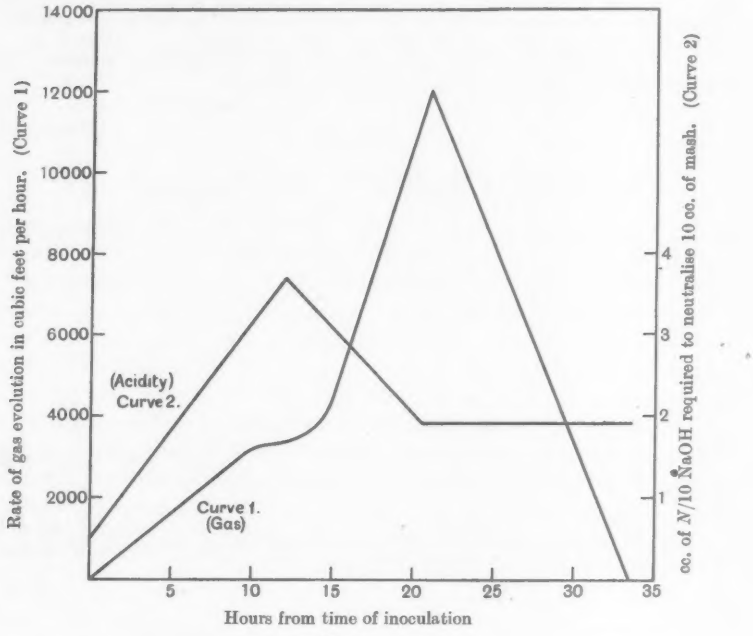


Fig. 1.

RATE OF GAS EVOLUTION, AND RISE AND FALL OF ACIDITY THROUGHOUT THE FERMENTATION.

The curves in Fig. 1 show the changes in

(1) the rate of gas evolution, and

(2) the acidity during a fermentation. The figures are the average of 12 fermentations of 6·5 % maize mash (40,000 gallons) with 4·7 % of inoculant.

The acidity is measured in cc. of *N*/10 alkali required to neutralise 10 cc. of mash after boiling the solution to remove carbon dioxide. Phenolphthalein is used as indicator.

It will be noted that the rate of gas evolution rises steadily with the increase of acidity for some time; it then becomes constant (in some cases it even slackens somewhat). As the acidity falls, the rate of gas evolution rises quickly to a maximum, and then falls rapidly until the end of the fermentation. Readings of the gas evolution were taken every hour. The acidity was estimated every three hours. The acidity at the end of the fermentation is generally higher than that of the mash when inoculated.

Composition of Gas.

Estimations were made of the composition of the gas evolved during fermentation. The following Table shows the results of one of these experiments.

The fermenting vessel was about two-thirds full of mash, the space above the latter being occupied by air before inoculation.

Table II.

Time 28.vi.'16	Gas evolved cubic feet per hour	CO_2 %	H_2 %	Air %	
4 p.m.	—	—	—	100	fermentation started
7 „	253	11·5	38·5	50	
8 „	834	27·1	57·9	15	
9 „	822	40·3	55·2	4·5	
10 „	660	46·0	59·0	3·0	
11 „	760	50·3	47·2	2·5	
29.vi.'16					
9.30 a.m.	1186	62	38	—	

The percentage of carbon dioxide did not vary from 9.30 a.m. 29. vi. '16 to the end of the fermentation, which lasted 36 hours. Total gas evolved 42,694 cubic feet. The high percentage of hydrogen in the gas evolved during the early hours is probably due to the greater solubility of the carbon dioxide.

The production of acetone and *n*-butyl alcohol at various stages in the fermentation is shown in Table III.

Table III.

Time	Acidity	Ratio, acetic to butyric acid	Amount of acetone and *n*-butyl alcohol mixture in 1 litre of mash
9.0 p.m.	1·0	1 : 0·5	None
11.0 „	1·5	1 : 0·62	None
12.15 a.m.	2·0	1 : 0·9	Trace
5.30 „	3·7	1 : 1·25	1 cc.
3.15 p.m.	2·0	1 : 0·28	4 cc.
4.30 „	1·6	1 : 0·25	5 cc.

Acidity is expressed in cc. of *N*/10 alkali required to neutralise 10 cc. of mash.

There appears to be an intimate connection between the production of acetone and *n*-butyl alcohol, and the fluctuations of acidity and rate of gas evolution.

Composition of Acids produced during Fermentation.

The acids present in the mash were examined in order to ascertain their nature and to determine if possible their function in the fermentation. The greater part of these acids was found to be volatile in steam. To study their nature the volatile acids were removed by distillation in a current of steam. The neutralised distillate was concentrated. The acids regenerated from the

concentrated solution were found by Duclaux's method [1895] to be butyric and acetic acids. By fractional crystallisation of the barium salts of the mixed acids, a separation was effected into two acids of different molecular weights. The barium content of each of the two fractions agreed with that for barium acetate and barium butyrate respectively. Neither by the Duclaux method of distillation nor by fractional crystallisation of the barium salts was any indication obtained of the presence of a third volatile acid in more than traces.

Fermentation in presence of Calcium Carbonate.

The presence of acetic and butyric acids in the fermenting mash is significant. It lends support to the hypothesis that these acids are intermediate products in the formation of acetone and *n*-butyl alcohol from the carbohydrate of the mash. A fermentation was carried out in the presence of calcium carbonate to determine the effect of neutralising the acids as produced. Under these conditions the production of acetone and *n*-butyl alcohol was almost entirely suppressed, and the calcium salts of acetic and butyric acids were produced usually in the ratio of 1 molecular proportion of acetic acid to 1·8 molecular proportions of butyric acid. Evidence was also obtained of a third acid only very slightly volatile in steam, having a molecular weight equal to or greater than that of butyric acid. The relative proportions of acetic and butyric acids vary under certain conditions and in one experiment a ratio of ten molecules of acetic to nine molecules of butyric acid was obtained.

If it be assumed that two molecules of acetic acid yield one molecule of acetone, and that each molecule of butyric acid yields by reduction one molecule of *n*-butyl alcohol, then the ratio of acetic acid to butyric acid, when the fermentation is conducted in presence of calcium carbonate, should be 1 mol. : 1 mol., provided the balance of the reactions taking place is not upset in these circumstances. The result obtained in practice however is usually 1 molecular proportion of acetic acid to 1·8 molecular proportions of butyric acid.

RELATIVE RATE OF PRODUCTION OF ACETIC AND BUTYRIC ACIDS IN THE COURSE OF FERMENTATION.

Further information on the formation of the acids was obtained by determining the relative amounts of acids present at intervals throughout the fermentation. Shortly after inoculation acetic acid is found to be present in considerable amount compared with butyric acid, but as the acidity increases the relative proportion of butyric acid also increases until at the point of maximum acidity the ratio is about 1·1–1·5 molecular proportions of butyric acid to 1 molecular proportion of acetic acid.

From this point onwards the ratio of butyric acid to acetic acid falls progressively, until at the end of the fermentation the acids are in the ratio

of 4–5 molecular proportions of acetic acid to 1 molecular proportion of butyric acid. (See Summary of Results, p. 246, and Fig. 2.)

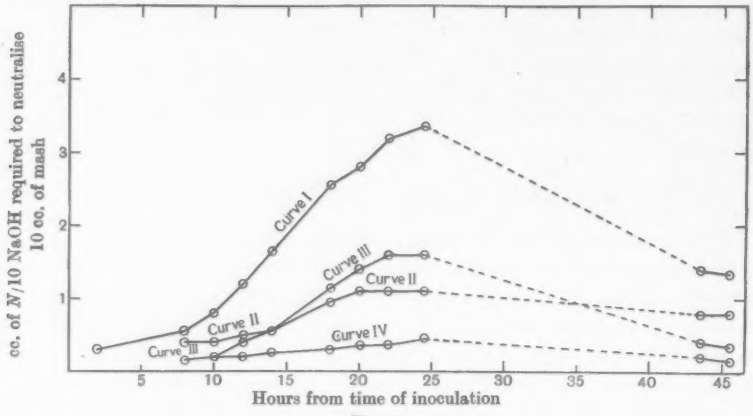


Fig. 2.

Explanatory:

Curve I. Total acidity of mash expressed in cc. $N/10$ NaOH per 10 cc. of mash.
Curve II. Acidity due to acetic acid.
Curve III. Acidity due to butyric acid.
Curve IV. Acidity due to slightly volatile acid.

A comparison of (*a*) the relative proportions of the two volatile acids in the mash at different periods and of (*b*) rise and fall of total acidity shows that during the production of acetone and *n*-butyl alcohol the proportion of butyric acid falls more rapidly than that of acetic acid, agreeing with the formation of a greater proportion of *n*-butyl alcohol than of acetone.

The addition of Acetic Acid and of other Acids to the Fermenting Mash.

When acetic acid is added to the fermenting mash an increase takes place in the amount of acetone obtained at the end of the fermentation.

A few preliminary experiments have been made in which other acids were added to the fermenting mash. Formic acid and trichloracetic acid tended to inhibit the fermentation. Propionic and butyric acids were converted into the corresponding alcohols. Valeric acid appeared to have no effect. The addition of aceto-acetic ester resulted in an increased yield of acetone.

Production of Ethyl Alcohol.

A small proportion of ethyl alcohol is usually obtained. It is doubtful whether it is a normal product of the fermentation of the carbohydrate, or a by-product derived from non-carbohydrate material in the mash. The possibility of its production by foreign organisms is not excluded. When infection of the mash has been observed, this has generally been due to lactic acid-producing organisms.

The Chemical Changes involved in the Fermentation.

The acetic acid normally formed in the mash appears to yield acetone, for on neutralising the acid by means of calcium carbonate only traces of acetone are obtained. As already mentioned, the addition of acetic acid to the mash (fermenting in absence of $CaCO_3$) causes an increase in the yield of acetone. This action might be compared to the production of acetone by distilling calcium acetate, carbon dioxide and water being produced instead of calcium carbonate. The acetic acid may on the other hand be supposed to undergo a condensation to aceto-acetic acid, which subsequently produces acetone and carbon dioxide.

A tentative suggestion to explain the peculiar action on acetic acid is to consider acetic acid as a monoketide, $H.CH_2.CO.OH$. By condensation $CH_2 : CO$ yields

$$CH_2{:}C(OH)—CH{:}CO.$$

The elimination of CO_2 and the addition of the elements of water would then yield acetone.

$$.CH_2{:}C(OH)—CH{:}CO + H_2O \rightarrow CH_3.CO.CH_3 + CO_2.$$

Aceto-acetic acid is a diketide

$$H.CH_2{:}C(OH)—CH.CO.OH \rightarrow CH_2{:}C(OH)—CH{:}CO + H_2O \rightarrow CH_3.CO.CH_3 + CO_2.$$

This hypothesis may be extended and is capable of explaining ordinary alcoholic fermentation.

In these reactions acetone and carbon dioxide are produced in the ratio of 58 to 44 by weight. It is obvious therefore from the relative amounts of acetone and carbon dioxide in the end-products of the fermentation that there must be some additional source of carbon dioxide. The 10·77 parts of acetone (see Table I) are only equivalent to 8·2 parts of carbon dioxide, but the total of the latter obtained is 62·61 parts. By comparing Curve 1, Fig. 1 and Table III, it will be seen that a large volume of gas has been evolved before any appreciable amount of acetone or *n*-butyl alcohol has been produced. On the large scale it has been observed that from 20 % to 30 % of the total volume of gas is evolved before the maximum acidity is reached, and the production of acetone and *n*-butyl alcohol in appreciable amounts begins.

A careful estimation of the total acids, carbon dioxide and hydrogen formed when the fermentation is carried out in presence of calcium carbonate, may throw light on the source of the carbon dioxide and hydrogen. All that can be said in this connection, without further experiments, is, that the weights of acetic and butyric acids calculated from the weight of mixed calcium salts obtained when a fermentation was carried out in presence of calcium carbonate were equivalent to only a part of the starch fermented. No measurements of the gas evolved nor of its composition have been made.

The proportions of the end-products obtained and their relation to the amount of carbohydrate fermented may be fairly accurately represented by the following two equations:

$$3\,C_6H_{10}O_5 + 3\,H_2O = 3\,C_6H_{12}O_6.$$
$$3\,C_6H_{12}O_6 = 2\,C_4H_{10}O + C_3H_6O + 7\,CO_2 + 4\,H_2 + H_2O.$$

The amounts of the end-products calculated as percentages on the starch fermented from the equations and actually obtained are as follows:

	From the equations	Found (Table I)
Acetone	11·9	10·77
n-Butyl alcohol	31·0	25·07
Carbon dioxide	63·0	62·61
Hydrogen	1·6	1·60
		(1·80) residual acidity

The nature of the reactions taking place to produce these results is obscure. Apparently acetic and butyric acids are intermediate products in the formation of acetone and *n*-butyl alcohol. No information has been obtained concerning the source of the acetic and butyric acids or of the hydrogen and carbon dioxide. A portion of the last-named substance may arise from the conversion of the acetic acid into acetone. The following theories may account for the origin of the carbon dioxide and hydrogen and of part of the *n*-butyl alcohol, but do not explain the production of acetic acid.

Buchner and Meisenheimer [1908], in dealing with a fermentation yielding butyric acid and butyl alcohol together with other products, suggested that acetaldehyde was first produced, which yielded aldol by condensation. The butyl alcohol was obtained by reduction from aldol, while rearrangement of the aldol yielded butyric acid thus:

A. $2\,CH_3.CHO = CH_3.CH(OH).CH_2.CHO.$

$$CH_3.CH(OH).CH_2.CHO + 2\,H_2 = CH_3.CH_2.CH_2.CH_2.OH + H_2O.$$

B. $2\,CH_3.CHO \rightarrow CH_3.CH(OH).CH_2.CHO \rightarrow CH_3.CH_2.CH_2.COOH.$

A similar theory has been put forward by Harden [1901], and Grey [1914], to explain the mechanism of the fermentation produced by *B. coli communis*. According to this theory the carbohydrate is first decomposed into an intermediate substance which gives rise to formic acid and acetaldehyde. The former is converted into carbon dioxide and hydrogen; the latter undergoes the Cannizzaro reaction and yields ethyl alcohol and acetic acid.

The following equations show the application of the above-mentioned theories to the acetone fermentation:

$$3\,C_6H_{10}O_5 + 3\,H_2O = 3\,C_6H_{12}O_6$$

(Intermediate substance) $\qquad 6\,[C_3H_6O_3]$

$$6\,H.COOH \qquad 6\,CH_3.CHO$$

No direct evidence has yet been found of the formation of acetaldehyde or of formic acid in the acetone fermentation. It may, however, be pointed

out that the following empirical equations, in which the end-products are underlined, agree closely with the quantities actually found.

1. $6\,H.COOH = 6\,\underline{CO_2} + 4\,\underline{H_2} + 2\,H_2$;
2. $2\,C_2H_4O + 2\,H_2 = \underline{CH_3.CH_2.CH_2.CH_2.OH} + \underline{H_2O}$;
3. $4\,C_2H_4O = \underline{C_2H_6O} + \underline{CH_3.CH_2.CH_2.OH} + \underline{CO_2}$.

Many bacteria are known to produce the change represented in equation (1), but the organism employed in this work has not been tested in this respect. Equation (2) corresponds with Buchner's theory of the formation of butyl alcohol. As to the mechanism of equation (3) nothing is known.

The fact that formic acid was not found when the fermentation was carried out in presence of calcium carbonate is not necessarily a proof that this theory is incorrect. It has been shown by Pakes and Jollyman [1901] and Grey [1914] that formic acid and formates are decomposed by *B. coli* and possibly the acetone-producing organism may act similarly. It is intended to make experiments to discover whether this is the case by the addition of formates to the fermenting mash. Careful measurements of the composition of the gas evolved when the fermentation takes place in presence of calcium carbonate are also necessary, and the effect of additions of acetaldehyde should be studied.

The possibility of the production of *n*-butyl alcohol from amino-acids is excluded, as the quantity of amino-acids present is totally insufficient to account for the quantity of alcohol produced.

Experimental.

Investigation of Acids produced during the Acetone: n-Butyl Alcohol Fermentation Process.

A portion of the mash was subjected to a steam distillation. 250 cc. of liquid, having an acidity equivalent to 43·8 cc. $N/10$ alkali, gave 450 cc. of distillate having an acidity equal to 27·4 cc. $N/10$ alkali. The presence therefore of a volatile acid or acids is evident.

In order to obtain larger quantities of the volatile acids free from sugars and other impurities present in the fermentation liquid, a larger quantity of the mash was steam distilled. The distillate was neutralised by means of a saturated solution of barium hydroxide and the solution evaporated to a small bulk. A stream of carbon dioxide was passed through the solution to precipitate any slight excess of barium hydroxide, and the solution was next raised to boiling point, filtered and afterwards evaporated to dryness. During evaporation of the neutralised distillate it became darker in colour, changing from light to a darker brown. The barium salt was usually obtained as an amorphous brown mass which only crystallised with difficulty. On warming with dilute sulphuric acid an odour resembling that of acetic acid was obtained. 0·1957 g. of salt dried in the oven gave 0·1599 g. of $BaSO_4$; Ba = 48·1 %.

With another sample, 0·1500 g. gave 0·1206 $BaSO_4$; Ba = 47·3 %.

The steam distillate of a sample of fermented acetone mash usually gave a barium salt which only crystallised slowly. In one experiment however a crystalline product was readily obtained and after one crystallisation it yielded a white salt which contained 45·85 % of Ba. By crystallising the mother-liquors a salt was obtained in the form of plates which contained 49·90 % of Ba, and on further crystallisation the barium content was reduced to 45·57 %. The barium salt on warming with dilute sulphuric acid gave an odour somewhat similar to acetic acid and the free acid was stable to oxidising agents and bromine water.

The quantity of material available was not sufficient for further crystallisations but from the general properties of the compound it would appear to be mainly barium butyrate (requires Ba = 44·1 %). The other barium salt, if it is a salt of a fatty acid, would appear to be an acetate rather than a propionate, for even by crystallising the mother-liquors containing a relatively high percentage of the butyrate, a salt is obtained containing 49·9 % of Ba (barium propionate requires Ba = 48·4 %; barium acetate, Ba = 53·7 %; barium formate, Ba = 60·35 %).

Another crystallisation brings the barium content of this salt nearer to that of barium butyrate owing to the somewhat smaller solubility of this salt in water than of the acetate. These figures indicate the probable absence of a large proportion of barium propionate. It should be noted that a phenylhydrazine compound was obtained as described below. On heating the crude crystalline barium salt under diminished pressure with syrupy phosphoric acid, an acid distillate was obtained which gave with phenylhydrazine a small amount of a white precipitate which was insoluble in water and ether, but soluble in absolute alcohol. It was purified by dissolving in absolute alcohol and precipitating with ether. It melted at 235–238°, yielding a brown oil. Evaporation of the alcoholic solution mentioned above gave the substance in the form of small needles. In order to determine if there were any β-hydroxybutyric acid present the crude barium salt was dissolved in a small amount of water, the barium removed by the addition of slight excess of sulphuric acid, and the solution extracted several times with ethyl acetate. On evaporating the solvent a thick brown oil remained which did not solidify on cooling. It had an acid reaction and was stable towards bromine water and Fehling's solution, but was decomposed by chromic acid and concentrated sulphuric acid. It yielded the iodoform reaction with sodium hydroxide and iodine and also dissolved silver oxide, yielding a solution which rapidly darkened on exposure to light. Extraction with amyl acetate instead of ethyl acetate yielded an oil similar in properties to that described above. An acetone extraction followed by a salting out with calcium chloride gave a solution which contained some mineral matter. On evaporation to dryness followed by a further extraction with acetone or ether a light coloured oil was obtained. By acidifying the barium salt with sulphuric

acid, absorbing the mixture in anhydrous sodium sulphate, powdering and extracting with ether in a Soxhlet, a brown ethereal extract was obtained which had an odour similar to butyric acid. After removing most of the volatile fatty acids, the residue was tested for β-hydroxybutyric acid according to the method of Black [1908] using hydrogen peroxide and ferric chloride, and also by oxidising it with dichromate in presence of a mercuric salt. Neither method gave any positive result. These experiments would indicate the absence of β-hydroxybutyric acid.

In order to identify the volatile acids present, the barium salt dissolved in water was treated with the calculated amount of $N/1$ sulphuric acid to precipitate the barium, filtered from barium sulphate, and distilled according to Duclaux's method:

Vol. of distillate c.c.	$N/10$ KOH cc.	Percentage	Calculated for 1 mol. acetic acid: 1 mol. butyric acid
30	19·75	35·0	35·2
40	25·57	45·3	46·0
50	31·17	55·3	55·7
60	36·35	64·4	65·0
70	41·45	73·5	73·7
80	46·33	82·1	82·2
100	56·43	100·0	—

The results of the Duclaux estimation would appear to confirm our original observations that acetic and butyric acids were the only volatile acids present. The ratio of acetic acid to butyric was also determined by Dyer's method [1917]. About 1 gram of the salt dissolved in water was treated with a slight excess of $N/1$ sulphuric acid, the volume made up to 150 cc. and steam distilled.

The first 100 cc. of distillate required 29·52 cc. $N/10$ alkali for neutralisation and the second 100 cc. 14·9 cc. The distilling constant is 49·5.

Dyer's figures give 1 mol. acetic acid, 0·94 mol. butyric acid.

Stein's figures [1913] give 1 mol. acetic acid, 0·78 mol. butyric acid.

10 g. of the finely powdered barium salt were treated with a slight excess of phosphoric acid and distilled. The distillate was dried over phosphorus pentoxide and again distilled. It was collected in two fractions – 130° and 130–160°. Each fraction was then submitted to a Duclaux distillation.

Fraction – 130°			Fraction 130—160°		
Vol. of distillate cc.	$N/10$ KOH cc.	Percentage	Vol. of distillate cc.	$N/10$ KOH cc.	Percentage
30	4·75	34·4	30	19·95	38·1
40	6·18	44·8	40	25·45	48·5
50	7·53	54·6	50	31·15	59·4
60	8·90	64·5	60	35·85	68·3
70	10·05	72·8	70	40·55	77·3
80	11·25	81·5	80	44·75	85·3
100	13·80	100·0	100	52·47	100·0
1 mol. of acetic acid to 0·83 mol. of butyric acid.			1 mol. of acetic acid to 1·8 mols. of butyric acid.		

Thus by distilling the volatile acids and by collecting the distillate in two fractions the ratio in which the acids are present has been altered, and no appreciable amount of a third volatile acid was shown to be present.

Fermentation in presence of Calcium Carbonate.

For further examination of the acid produced, the fermentation was allowed to proceed in presence of calcium carbonate. A quantity of 2 litres of 5 % maize mash, sterilised at 30 lb. pressure for 2 hours, was inoculated with 2 % acetone inoculant. A slight excess of calcium carbonate was added, and the acidity was prevented from developing by the frequent addition of calcium carbonate, during the fermentation. Throughout the fermentation, the organism, examined microscopically, appeared normal. After 7 days' fermentation the suspended matter was removed, and the filtrate reserved for the examination of the calcium salts. 300 cc. of the filtrate were distilled, and the first 50 cc. of distillate shaken with an excess of anhydrous potassium carbonate. On "salting out," about 2 cc. of an oil were obtained. It had an odour similar to *n*-butyl alcohol and the oil also gave the iodoform test, when both sodium carbonate and ammonia were used.

For a further examination of its properties a fresh amount of calcium salt was made: 2 litres of 5 % maize mash were prepared as in the previous case. To this was added 40 cc. of inoculant and an excess of calcium carbonate. Probably the excess of calcium carbonate was too great, since the fermentation was not very satisfactory until a further amount of 40 cc. of acetone inoculant had been added. The fermentation was allowed to proceed for 7 days, and the liquid neutralised periodically by the addition of small amounts of calcium carbonate. The soluble calcium salts were filtered off, the insoluble portion washed with water and the washings added to the above solution. On evaporating to dryness (on the water-bath) the solution containing the calcium salts, 45 g. of solid were obtained (containing $H_2O = 7{\cdot}6\%$, $Ca = 17{\cdot}7\ \%$). The amount of sugar present was very small.

10 g. were distilled with an excess of glacial phosphoric acid---there was some charring and frothing. The distillate was strongly acid and was dried with calcium chloride and finally with phosphorus pentoxide. On redistillation it gave a fraction at 115–140° smelling strongly of acetic acid, whilst at 140–160° a second fraction was obtained, having an odour similar to butyric acid. From these two fractions the barium salts were prepared by neutralising with barium carbonate, filtering, and evaporating to dryness. The lower boiling fraction yielded a barium salt which contained $Ba = 47{\cdot}95\ \%$. On extracting the anhydrous salt several times with absolute alcohol to remove higher fatty acids if present, the barium content was increased to 51·57 %. By recrystallising the barium salt of the higher boiling fraction from water a salt was obtained containing 45·99 % Ba.

The results obtained by the fractional crystallisation of the barium salts indicate the presence of butyric and acetic acids. This was confirmed by a

Duclaux estimation. A weighed amount of the original calcium salt was dissolved in water, decomposed by the calculated amount of sulphuric acid and distilled:

Vol. of distillate cc.	N/10 KOH cc.	Percentage	Ratio, acetic acid : butyric acid
20	11·12	26·3	1 : 1·7
30	16·12	38·2	1 : 1·7
40	20·90	49·5	1 : 1·8
60	28·70	68·0	1 : 1·8
70	32·85	77·8	1 : 1·9
100	42·20	100·0	—
		Average ...	1 : 1·8
Ratio, acetic acid : butyric acid ...			1 : 1·8 mols. 1 : 2·6 by weight.

The ratio was also determined from the constants given by Dyer and by Stein [1913]. For this purpose two determinations were made. From the mean of these 58·3 % of the total acid distilled over in the first 100 cc. of distillate, giving from Dyer's constants, 1 mol. acetic acid : 2·4 mols. butyric acid; from Stein's constants, 1 mol. acetic acid : 2·0 mols. butyric acid.

In order to check these methods, 1 g. of calcium salt, after acidifying with $N H_2SO_4$, was steam distilled, the distillate being neutralised with a saturated solution of barium hydroxide. When all the volatile acid had been driven over, the neutralised distillate was evaporated to a small bulk. A stream of carbon dioxide was passed through the solution to remove any slight excess of barium hydroxide: the insoluble barium carbonate was removed by filtration after boiling, the filtrate evaporated to dryness and the residue heated in an oven until constant in weight. 0·1500 g. gave 0·1202 g. $BaSO_4$; Ba = 47·20 %.

This figure corresponds with the presence of acetic acid and butyric acid in the ratio 1 mol. acetic acid to 1·75 mols. of butyric acid.

Summary.

Method	Dyer	Stein	Duclaux	Ba salt
Ratio, acetic acid : butyric acid, mols.	1 : 2·4	1 : 2·0	1 : 1·8	1 : 1·8
Ratio, acetic acid : butyric acid, by weight ...	1 : 3·5	1 : 2·9	1 : 2·6	1 : 2·6

Disregarding the Dyer determinations it would seem as if the proportion in which acetic and butyric acids were present is approximately 1 : 1·8 (mol.) or 1 : 2·6 (weight). It should be pointed out that the above workers carried out their investigations on prepared mixtures of the pure acids. In our investigation, the mash liquor and a non-volatile acid have probably some effects on the distillation figures and the results may not be so uniform. In some other experiments higher proportions of acetic acid were obtained. Evidence was also obtained of the presence of a third acid only slightly volatile in steam.

1 g. of the calcium salt was dissolved in a small amount of water, and a solution of 4 g. of quinine sulphate dissolved in absolute alcohol added. After heating for a short time to 100° the mixture was allowed to stand for a few days. The precipitate was removed by filtration and the filtrate evaporated to dryness in vacuo and finally extracted with chloroform.

Although the quinine salts have not yet been examined in detail, a preliminary investigation points to the presence of another acid in addition to acetic and butyric acids. Some other evidence on this point is given later.

Nature of the Acids present during the course of a Normal Fermentation.

In order to investigate the nature of these acids samples were withdrawn periodically from a fermentation and a Duclaux estimation was carried out directly on the mash after determining the total acidity. This method was adopted as it was imperative to carry out all the determinations on the same fermentation, and it appeared to yield the most concordant results, while the time required to carry out a determination was short. The results are comparable among themselves, since the influence of substances in the solution is approximately constant. The suspension of carbohydrate material was allowed to settle, as far as possible, and the supernatant liquid decanted through purified glass-wool to remove any further amount of suspended matter. This investigation was only undertaken in the case of one or two fermentations and the results obtained may not be general. The following figures are therefore of the nature of a preliminary investigation.

In order to test the accuracy of the results obtained by direct distillation of the mash two experiments were carried out with the same sample of mash as follows:

A Duclaux estimation was carried out on the mash.

Acidity of mash, 5·1 cc. $N/10$ [1].

Duclaux estimation				
Vol. of distillate cc.	$N/10$ KOH cc.	Percentage		
10	4·90	12·6	1·0	
20	9·48	24·1	0·9	
30	13·93	35·7	1·1	
40	18·03	46·3	1·1	Ratio acetic : butyric 1 : 1·0
50	21·86	56·1	1·1	
60	25·41	65·2	1·0	
80	31·90	81·9	0·9	
100	38·96	—	—	

Another portion was distilled in steam at about 60° under diminished pressure. The distillate was neutralised and concentrated; the calculated amount of sulphuric acid added, and the ratio of butyric and acetic acids determined by distillation at constant volume by a method which has been recently described by Reilly and Hickinbottom [1919].

[1] The values recorded for the acidity of the mash refer to the number of cc. of $N/10$ NaOH required to neutralise 10 cc. of mash.

200 cc. of mash on steam distillation gave a distillate requiring 89·0 cc. $N/10$ $Ba(OH)_2$ for neutralisation. The volatile acid is 87 % of the total acidity.

Estimation of ratio of acetic and butyric acids by new method.

Vol. of distillate cc.	Percentage of total acid distilled	Ratio	
		acetic acid	butyric acid
20	16·5	1	1·14
30	23·3	1	1·15
40	29·2	1	1·10
50	35·1	1	1·14
60	40·1	1	1·13
70	45·7	1	1·13
80	48·7	1	1·12
90	52·4	1	1·13
100	55·6	1	1·10
		Av. 1	1·12

The agreement between the two series is good. The difference is not more than that due to experimental error in determining the ratio by the Duclaux method.

The estimation on the mash was checked by distilling 1 litre to a small bulk, when it was diluted with distilled water, and again distilled. The distillate so obtained was neutralised with normal sodium hydroxide solution and evaporated to a small bulk. The volatile acids in the neutralised concentrated liquor were estimated by Dyer's method and the results calculated, using both Stein's and Dyer's figures.

During the removal of the volatile fatty acids from the mash, it is extremely difficult to distil all the acetic acid over without carbonising the residue and consequently the results from the Dyer estimations may be somewhat too low for acetic acid.

Sample I ... ... 7.30 p.m.
Acidity ... ... 0·33 cc. $N/10$
Sample II... ... 9.30 p.m.
Acidity ... ... 0·28 cc. $N/10$
Sample III ... 11.30 p.m.
Acidity ... ... 0·32 cc. $N/10$

The composition of the mixture of acids present in these three samples was not estimated

Sample IV ... 1.30 p.m.
Acidity ... ... 0·51 cc. $N/10$

Duclaux estimation

Vol. of distillate cc.	$N/10$ KOH cc.	Percentage	Calc. for 1 mol. acetic acid : 0·33 mol. butyric acid
30	1·51	29·7	29·4
40	1·98	39·0	39·0
50	2·47	48·6	48·8
60	2·95	58·1	57·7
80	3·85	75·8	77·0
100	5·08	100·0	—

Sample V ... 3.30 a.m.
Acidity... ... 0·77 cc. *N*/10

Duclaux estimation

Vol. of distillate cc.	N/10 KOH cc.	Percentage	Calc. for acetic acid 1 mol. : butyric acid 0·5 mol.
30	1·85	31·8	31·4
40	2·42	41·6	41·3
50	2·97	51·0	50·8
60	3·51	60·3	60·2
70	4·10	70·5	69·4
80	4·62	79·4	78·8
100	5·82	100·0	—

Sample VI ... 5.30 a.m.
Acidity... ... 1·20 cc. *N*/10

Duclaux estimation

			Ratio, acetic acid : butyric acid	
30	3·15	33·7	1 : 0·71	
40	4·15	44·4	1 : 0·71	
50	5·09	54·5	1 : 0·84	The average proportion is 1 mol. of acetic acid : 0·83 mol. of butyric acid
60	5·94	63·6	1 : 0·77	
70	6·89	73·8	1 : 1·0	
80	7·67	82·1	1 : 1·0	
100	9·34	100·0	—	

Sample VII ... 7.30 a.m.
Acidity... ... 1·63 cc. *N*/10

Duclaux estimation

			Percentage calc. for 1 mol. acetic acid : 1 mol. butyric acid
30	4·58	34·6	35·2
40	6·01	45·5	46·0
50	7·31	55·3	55·7
60	8·60	65·1	65·0
70	9·80	74·1	73·7
80	11·01	83·3	82·2
100	13·22	100·0	—

Sample VIII ... 11.30 a.m.
Acidity... ... 2·55 cc. *N*/10

Duclaux estimation

			Ratio, acetic acid : butyric acid	
30	7·53	35·7	1 : 1·1	
50	11·93	56·5	1 : 1·2	
60	13·89	65·8	1 : 1·2	1 mol. of acetic acid to 1·2 mols. of butyric acid
70	15·78	74·8	1 : 1·2	
80	17·55	83·1	1 : 1·3	
100	21·10	100·0	—	

Sample IX ... 1.40 p.m.
Acidity... ... 2·80 cc. *N*/10

Duclaux estimation

Vol. of distillate cc.	*N*/10 KOH cc.	Percentage	Ratio, acetic acid : butyric acid	
30	8·78	36·0	1 : 1·2	
40	11·60	47·6	1 : 1·3	
50	14·00	57·4	1 : 1·3	1 mol. of acetic acid to 1·30 mols. of butyric acid
60	16·30	66·8	1 : 1·3	
70	18·45	75·6	1 : 1·4	
80	20·50	84·0	1 : 1·5	
100	24·40	100·0	—	

A Dyer estimation of the distillate from 1000 cc. required
27·8 cc. *N*/10 KOH to neutralise acid in 1st 100 cc.
12·45 „ „ „ 2nd „
These figures give a distilling constant of 55·2 which from
Dyer's figures give a ratio of 1 mol. of acetic acid : 1·7 mols. of butyric acid.
Stein's „ „ „ „ „ „ 1·4 „ „ „

Sample X ... 3.30 p.m.
Acidity... ... 3·25 cc. *N*/10

Duclaux estimation

			Ratio, acetic acid : butyric acid	
30	9·68	36·1	1 : 1·2	
40	12·78	47·6	1 : 1·3	
50	15·65	58·3	1 : 1·3	
60	18·10	67·4	1 : 1·5	1 mol. of acetic acid to 1·4 mols. of butyric acid
70	20·40	76·0	1 : 1·5	
80	22·61	84·2	1 : 1·6	
100	26·85	100·0	—	

A Dyer estimation on the distillate from 1000 cc. required
52·6 cc. *N*/10 KOH to neutralise the acid in 1st 100 cc.
23·8 „ „ „ „ 2nd „
Distilling constant = 54·8.
Dyer's figures give a ratio of 1 mol. of acetic acid : 1·6 mols. of butyric acid.
Stein's „ „ „ „ „ „ 1·4 „ „ „

Sample XI ... 6.0 p.m.
Acidity... ... 3·35 cc. *N*/10

Duclaux estimation

			Ratio, acetic acid : butyric acid	
30	10·10	35·9	1 : 1·2	
40	13·20	47·0	1 : 1·2	
50	16·00	56·9	1 : 1·3	
60	18·72	66·6	1 : 1·3	1 mol. of acetic acid to 1·3 mols. of butyric acid
70	21·30	75·9	1 : 1·5	
80	23·60	84·0	1 : 1·5	
100	28·10	100·0	—	

A Dyer estimation on the distillate from 1000 cc. required
33·0 cc. *N*/10 alkali to neutralise acid in 1st 100 cc.
14·0 „ „ „ „ 2nd „
Distilling constant = 57·6.
Dyer's figures give a ratio of 1 mol. of acetic acid : 2·1 mols. of butyric acid.
Stein's „ „ „ 1 „ „ 1·9 „ „

Sample XII ... 1.0 p.m.
Acidity... ... 1·4 cc. $N/10$

Duclaux estimation

Vol. of distillate cc.	$N/10$ KOH cc.	Percentage	
30	2·43	27·3	
40	3·35	37·6	
50	4·15	46·6	1 mol. of acetic acid to 0·22 mol. of butyric acid
60	4·98	56·0	
70	5·80	65·1	
80	6·75	75·8	
100	8·90	100·0	

A Dyer estimation on the distillate from 1000 cc. required
11·1 cc. $N/10$ alkali to neutralise acid in 1st 100 cc.
6·4 „ „ „ „ 2nd „
Distilling constant = 42·4.

Dyer's figures give the ratio 1 mol. of acetic acid : 0·40 mol. of butyric acid
Stein's „ „ „ 1 „ „ 0·33 „ „ „

Sample XIII ... 3.0 p.m.
Acidity ... ... 1·35 cc. $N/10$

Duclaux estimation

30	2·52	28·0	
40	3·45	38·3	
50	4·09	45·4	1 mol. of acetic acid to 0·2 mol. of butyric acid
60	4·85	53·9	
70	5·85	65·0	
80	6·71	74·5	
100	9·00	100·0	

Summary of Results.

Age of fermenting mash in hours	Acidity*	Ratio acetic acid : butyric acid as determined by method of: Duclaux Determined in the mash	Dyer Determined in the distillate from the mash	Duclaux Determined in the distillate from the mash	Stein Determined in the distillate from the mash
2	0·33	—	—	—	—
4	0·28	—	—	—	—
6	0·32	—	—	—	—
8	0·51	1 : 0·33	—	—	—
10	0·77	1 : 0·5	—	—	—
12	1·20	1 : 0·83	—	—	—
14	1·63	1 : 1	—	—	—
18	2·55	1 : 1·2	—	—	—
20	2·80	1 : 1·3	1 : 1·7	—	1 : 1·4
22	3·25	1 : 1·4	1 : 1·6	—	1 : 1·4
24½	3·35	1 : 1·3	1 : 2·1	—	1 : 1·9
43½	1·4	1 : 0·22	1 : 0·43	(1 : 0·4)	1 : 0·33
45½	1·35	1 : 0·20	—	—	—

* Expressed in cc. of $N/10$ alkali required to neutralise 10 cc. of mash.

To test the view that the production of "oil" and the diminution of butyric acid were connected, the amount of "oil" produced was determined on samples taken during the progress of a fermentation. The ratios in which the volatile acids were present in the same sample were also determined.

Time	Acidity	Ratio determined by Duclaux estimation, acetic acid : butyric acid	Volume of oil from one litre of mash
9.0 p.m.	1·0	1 : 0·5	None
11.0 „	1·5	1 : 0·62	„
12.15 a.m.	2·0	1 : 0·9	Trace
5.30 „	3·7	1 : 1·25	1 cc.
3.15 p.m.	2·0	1 : 0·28	4 „
4.30 „	1·6	1 : 0·25	5 „

These results indicate that traces of acetone and *n*-butyl alcohol are produced before the maximum acidity has been attained. The production of "oil" in the fermentation using calcium carbonate appears to confirm this result.

As far as can be judged from the limited number of experiments carried out, these ratios indicate that while the acidity of the mash is increasing previous to the production of *n*-butyl alcohol and acetone, the production of butyric acid proceeds at a faster rate than that of acetic acid. In the particular fermentation examined the maximum amount of butyric acid appears to be present at the time of maximum acidity, and when the production of acetone and *n*-butyl alcohol takes place, the amount of butyric acid diminishes. It would seem that the production of butyric and acetic acids, of *n*-butyl alcohol, and of acetone is intimately connected.

In the presence of calcium carbonate fermentation apparently proceeds as far as the point corresponding to the maximum acidity in a normal fermentation.

Evidence of the presence of a Third Acid only slightly volatile in Steam.

Indications have been obtained of the presence of another acid in the mash; it has not been isolated. In determining the ratio of butyric acid and acetic acid by Duclaux's method, it was observed that the amount of acid present in the original mash, calculated from Duclaux's table, did not agree with the total acid present, as estimated by titration.

The following tables and Fig. 2 show the percentage of the total acidity which is accounted for by butyric and acetic acids:

Acidity of the mash	Acetic and butyric acids, percentage of total acidity
1·0	105 (carbon dioxide present in addition
1·5	— to fatty acids)
2·0	90
3·7	86
2·0	70
1·6	74

Another mash gave the following results:

Age of fermenting mash in hours	Acidity of the mash	Acetic and butyric acids, percentage of total acidity
8	0·51	107 (carbon dioxide present)
10	0·77	80
12	1·20	80
14	1·63	83
18	2·55	84
20	2·80	89
22	3·25	83
24½	3·35	88
43½	1·4	70
45½	1·35	73

This agreement between two series points to the existence of an acid not readily volatile in steam which increases in proportion to the volatile acids after the maximum acidity has been reached. The calcium salt obtained by fermenting in presence of chalk, on distillation by the Dyer method, yields only about 53–55 % of its weight as volatile acid. The moisture and calcium oxide only account for about 30–35 % of the salt. The amount of sugar present in the crude salt was extremely small. There was, therefore, about 10–15 % not accounted for.

The quinine salt prepared from the calcium salt, on hydrolysis with baryta, gives a mixture of barium salts which points to the existence of another acid with an equivalent equal to or greater than that of butyric acid.

Addition of Acetic Acid to the Fermenting Mash. [See Desborough etc., 1918.]

105 lb. of maize meal were used to prepare 168 gallons of mash: the mash was inoculated with half a gallon of fermenting mash. Total volume of mash 168·5 gallons.

The acetic acid was added to the mash in the concentrated form, but dilute acid may equally well be used. During the addition of the acid the mash was vigorously stirred and the stirring was continued for 15 minutes after the acid had been added. The first lot of 500 cc. of acid was added 24 hours after inoculation, as soon as the normal acidity had passed its maximum and was beginning to fall.

Subsequent additions of 500 cc. of acid were made as soon as the acidity of the mash had fallen to the same point at which the first lot of acid was added. In all, seven lots of 500 cc. of acid were added, making a total of 3500 cc. of acid added.

Specific gravity of 97 % acetic acid used, 1·0625.

Total weight added, 3718·7 g.; so that 3607·1 g. of acetic acid were added.

The total acetone produced at the end of the fermentation was 27·27 g. per gallon. The volume of fermented mash at the end of the fermentation amounted to 163 gallons, as 4 gallons were taken out for a control im-

mediately after inoculation and 1·5 gallons were drawn off at intervals for estimating the acidity.

The total acetone produced was therefore 163 × 27·27 = 4445·01 g.

The acetone produced in the control to which acetic acid was not added amounted to 19·54 g. per gallon. The total acetone produced in 163 gallons of mash to which acetic acid was not added would be 163 × 19·54 g. = 3185·02 g.

Hence the acetone produced from the acetic acid = 4445·01 − 3185·02 = 1260 g.

Assuming that two molecules of acetic acid produce one molecule of acetone, the 3607 g. of acetic acid added should give 1743·4 g. of acetone. The amount actually found was 1260 g. or 72·2 % of the theoretical amount.

The yield would probably have been better had less acetic acid been added, as apparently more acetic acid was added to the mash than could be converted.

The acidity of the mash at the end of the fermentation remained abnormally high, 10 cc. of mash requiring 3·0 cc. *N*/10 alkali for neutralisation, instead of the normal amount of 1·9 cc.

In another experiment made about the same time, a similar result was obtained. In this case also, too much acetic acid was added, the yield of acetone was 79·9 % of the theoretical amount that should have been obtained from the total acetic acid added.

There were no indications of infection by lactic acid producing organisms in either of these fermentations. The volume of gas evolved was slightly above the normal.

A large scale experiment was carried out in which over a ton of acetic acid was added to 86,000 gallons of fermenting mash.

Unfortunately the mash became infected by lactic acid producing organisms before the fermentation was completed. In spite of this the weight of acetone produced was much above the normal amount, the excess of acetone obtained, over the normal yield from the grain, showing a yield of 61·27 % of the theoretical from the acetic acid added.

Examination of an Infected Mash.

A sample was taken from a fermentation in which the acidity had become high. The sample contained "oil" and had an acidity = 6·3 cc. *N*/10 NaOH. Part of the acidity was due to the presence of volatile acids, which were estimated by the Duclaux method.

Vol. of distillate cc.	*N*/10 KOH cc.	Percentage	
30	9·20	28·9	The distillation figures agree with a mixture of butyric and acetic acids in the ratio of approximately 3 mols. of acetic acid to 1 mol. of butyric acid
40	12·20	38·4	
50	15·30	48·2	
60	18·20	57·3	
70	21·35	67·3	
80	24·50	77·5	
100	31·75	100·0	

From this estimation approximately 55 % of the total acidity is accounted for as acetic and butyric acids. For the examination of the non-volatile acid, about 3 litres of the mash were evaporated down to a very small bulk. The residue, which was very dark in colour and sticky when cold, was mixed with fine silica while still hot, and finally made into a hard mass by stirring in finely-powdered anhydrous sodium sulphate. The mass was then broken up and extracted with ether in a Soxhlet extraction apparatus. A brown syrupy extract was obtained, which had an acid reaction. Oxidation by means of dilute potassium permanganate solution in presence of sulphuric acid yielded acetaldehyde. The brown liquid gave a *zinc* salt containing Zn = 26·6 %. (Zinc lactate requires Zn = 26·9 %.)

Another portion gave a *barium* salt which did not crystallise. The *calcium* salt crystallised in small nodules. A sample of the calcium salt after 3 crystallisations showed a slight laevo-rotation $[\alpha]_D = -0{\cdot}73°$; after 8 crystallisations $[\alpha]_D = -1{\cdot}34°$. The *copper* salt crystallised in long blue prisms.

By distilling the acid under moderately diminished pressure a solid distillate was obtained having a constant m.p. of 125° after 3 crystallisations from chloroform (lactide from lactic acid, m.p. 125°). A mixture with a specimen of pure lactide made from lactic acid melted at the same temperature. The acid cooled in ice was oxidised by the action of dilute hydrogen peroxide solution, in presence of a trace of ferrous acetate, in the manner described by Fenton and Jones [1900]. On the addition of a few drops of phenylhydrazine acetate solution, a red precipitate was produced which after washing well with water and small amounts of ether melted at 177–178° with decomposition. The melting point was not depressed by the addition of some pure pyruvic phenylhydrazone. The acid is therefore lactic acid.

A high acidity mash contains lactic, acetic and butyric acids.

Summary.

In the fermented mash, acetic and butyric acids are present in varying proportions. During the progress of the fermentation the ratio of butyric acid to acetic acid increases with the increase in the acidity of the mash, reaching a maximum when the acidity is at its highest. With the production of "oil" the ratio of butyric acid to acetic acid diminishes, until the mash contains an excess of acetic acid. If the acidity is neutralised by the addition of calcium carbonate, the calcium salts of the acids present in the mash replace the acetone and *n*-butyl alcohol, and these latter are produced in almost negligible amount. In the presence of calcium carbonate, however, the fermentation proceeds as far as the point corresponding to the maximum acidity in a normal fermentation. It is extremely probable that acetic and butyric acids are not the only acids present. The type of infection most frequently observed in the fermenting mash produces lactic acid. When the mash is

infected in this way the amount of volatile acid accounted for by the Duclaux estimation is lower than in the case of a normal fermentation.

When acetic acid is added to the fermenting mash an increased yield of acetone is obtained; the yield of *n*-butyl alcohol being unchanged. Propionic and butyric acids when added appear to be converted into the corresponding alcohols. An increased yield of acetone has been obtained by the addition of aceto-acetic ester.

In conclusion the authors take this opportunity of expressing their thanks to Capt. Desborough, R.A., Superintendent, and Mr W. T. Thomson, Manager of the Royal Naval Cordite Factory, Dorset, both for the interest they have taken in this investigation, and for granting permission to publish the results obtained. In addition we are indebted to Dr A. Harden for suggestions which may help to explain the mechanism of the reactions taking place.

REFERENCES.

Black (1908). *J. Biol. Chem.* **5**, 207.
Botkin (1893). *Zeitsch. Hyg.* **11**, 421.
Bredemann (1909). *Centr. Bakt. Par.* II, **23**, 385.
Buchner and Meisenheimer (1908). *Ber.* **41**, 1410.
Desborough and Others (1918). Eng. Patent 128403.
Duclaux (1895). *Ann. Inst. Past.* **9**, 269.
Dyer (1917). *J. Biol. Chem.* **28**, 445.
Farbenfabr. vorm F. Bayer & Co. German Patents 289687, 294683, 19143.
Fenton and Jones (1900). *J. Chem. Soc.* **77**, 71.
Fernbach and Strange (1912). Eng. Patent 21073.
Fitz (1878). *Ber.* **11**, 42.
—— (1882). *Ber.* **15**, 867.
—— (1883). *Ber.* **16**, 844.
—— (1884). *Ber.* **17**, 1188.
Grey (1914). *Proc. Roy. Soc.* B. **87**, 470, 472.
Grimbert (1893). *Ann. Inst. Past.* **7**, 353.
Gruber (1887). *Centr. Bakt. Par.* **1**, 367.
Harden (1901). *J. Chem. Soc.* **79**, 610.
Northrop (1919, 1). *J. Biol. Chem.* **39**, 1.
—— (1919, 2). U.S. Amer. Patent 1293172.
Pakes and Jollyman (1901). *J. Chem. Soc.* **79**, 386.
Perdrix (1891). *Ann. Inst. Past.* **5**, 287.
Reilly and Hickinbottom (1919). *Roy. Soc. Dublin*, **15**, 513.
Ricard (1918). Eng. Patent 130666.
Schardinger (1905). *Centr. Bakt. Par.* II, **14**, 772.
—— (1907). *Centr. Bakt. Par.* II, **18**, 748.
Stein (1913). *J. Prakt. Chem.* **88**, 83.
Weizmann (1915). Eng. Patent 4845.
—— (1919). U.S. Amer. Patent 1315585.

XXV. A NOTE ON BRAUNSTEIN'S MODIFICATION OF THE MÖRNER-SJÖQVIST PROCESS FOR THE ESTIMATION OF UREA.

By ALAN HERAPATH TODD.

From the Physiological Laboratory, Guy's Hospital.

(*Received March 8th, 1920.*)

An oft-repeated objection to the Mörner-Sjöqvist process is the fact that hippuric acid and creatinine escape precipitation, and appear in the final result as urea. Braunstein suggested that this might be avoided by using crystalline or syrupy phosphoric acid instead of sulphuric acid at the "Kjeldahl" stage, and as this proposal has gained added interest in view of the large amount of work now being done upon creatinine, the matter has been re-investigated.

The fundamental point in the whole process is the assumption that urea is completely incinerated under the conditions prescribed by Braunstein, viz., heating at 145° for 4½ hrs. To test this, an aqueous solution of known strength was used:

Expt. 1. Urea, re-crystallised from water, dried over H_2SO_4 *in vacuo*; solution contained N = 0·327 %, by ordinary Kjeldahl. 10 cc. heated in an oil-bath for various periods with Kahlbaum's crystalline phosphoric acid:

	Wt. acid used g.	Heated for hrs.	Temp. °C.	% N found	Remarks
(*a*)	10	4½	145	0·220	Braunstein's conditions
	—	—	—	0·220	
	—	—	—	0·261	
(*b*)	10	5	175	0·316	Results better, but N values
	—	—	—	0·317	still too low
	—	—	—	0·318	
(*c*)	10	6	178	0·318	No improvement with longer
	—	—	—	0·318	time
(*d*)	20	5	150	0·260	10 g. acid are enough
(*e*)	30	5	150	0·291	„ „

It is evident, from consideration of the three experiments included in Group (*a*), that urea-estimation is by no means quantitative under the original conditions. This might have been due to insufficiency of acid, too low a temperature, or too short a period of heating. The results of varying these conditions are seen in the experiments cited above in Groups (*b*), (*c*), (*d*), and (*e*), from which it appears that the temperature is the most important factor.

There remained the possibility that the small quantity of creatinine and other reducing bodies present in normal urine might in some way facilitate the process, so it was decided to incinerate entire urine with phosphoric acid and with sulphuric acid, and to compare the results.

Expt. 2. Normal urine, boiled and preserved with chloroform, N = 0·955 %, by sulphuric acid. 5 cc. used for each determination.

	Wt. acid used g.	Heated for hrs.	Temp. ° C.	% N found	Remarks
(*a*)	10	10	180	0·166	Acid insufficient
(*b*)	15	5	175	0·855	
	—	—	—	0·863	
	—	—	—	0·868	
(*c*)	15	10	180	0·896	
	—	—	—	0·900	
(*d*)	15	14	185	0·899	Liquid in flask after ignition
	—	—	—	0·903	was dark brown in colour,
	—	—	—	0·904	with white crystals on cooling

The last four values, though not equalling the figure for the total nitrogen, yet approximate so closely to one another as to suggest that the ignition of the urea is complete, and therefore that the ratio ·904/·955 represents the proportion of the urea-nitrogen to the total nitrogen. To decide whether this was the case or not, an "artificial urine" was made up, containing:

Creatine, dried at 100° and *in vacuo*, over H_2SO_4				N = 0·0233 %
Hippuric acid	,,	,,	,,	N = 0·0030
Urea	,,	,,	,,	N = 0·8937
				Total N is ∴ = 0·9200 %, both by calculation and by Kjeldahl; of which urea = 97·14%

(It was found impossible, in practice, to dry the urea to a constant weight, as was done with the other two substances; it was therefore dried until its loss of weight began to be uniform, and then the exact amount of it in solution was calculated from the difference between the total N and the (creatine + hippuric) N.)

Expt. 3. 5 cc. of the above artificial urine, with 15 g. of phosphoric acid, heated at 185° for 14 hrs.; the % N found was: 0·900, 0·882, 0·880, 0·873, 0·900, 0·895. *Average* = 0·892 %, *as compared with* 0·894, *calculated.*

The values given in the last group of six experiments are perhaps not quite as concordant as might be wished, but owing to the accidental destruction of notes in which other series of experiments were recorded, they are the only ones available at the moment. However, they are not far out, and if care be taken at the beginning of distillation, much better results can be obtained. *These figures prove that incineration of the Mörner-Sjöqvist filtrate for* 14 *hours at* 185° *with phosphoric acid gives an accurate, quantitative estimation of the urea, and the urea only.*

One possible fallacy remains, perhaps, and that is, the occurrence of a double compensatory error, a slight loss on the urea being repeatedly and exactly balanced by a slight incineration of the creatine and hippuric acid. This difficulty was readily disposed of by heating suitable quantities of these latter bodies with phosphoric acid under identical experimental conditions, when it was found that not a trace of incineration occurred.

In conclusion, it may be useful to add a few practical notes on the technique of the method. In the first place, the time taken in ignition is no objection to the process, since these experiments are always performed in a series, and so one day's material can be heated whilst another lot is being distilled and titrated. The actual heating is best performed in a bath of "government" colza oil, the height of the gas-flame being regulated by a regulator, so that, once adjusted, it is only necessary to turn the gas full on to secure the exact degree of heating required. It is best to incinerate in Jena flasks of the ordinary shape, so as to avoid transference before distillation, and as soon as ignition is completed, 100 cc. or more of distilled water should be added; if this is not done, a hard white mass forms on cooling, which it is most difficult to re-dissolve. Mica scales having been added, strong soda solution may be run in fairly quickly, as there is no heat evolved. As soon as the distillation-tube has been adjusted, the two layers of liquid should be gently mixed, and then be heated *very slowly* to boiling-point: in this way, much of the ammonia is driven over before rapid ebullition begins, and the rapid "surging" of the liquid, and the loss of ammonia from sudden mixing of the two hot liquids (that were so troublesome in the older method), are completely avoided. Lastly, it may be mentioned that the most convenient way of weighing out the crystalline phosphoric acid is to melt it in a water-oven, and then to pour it out into a weighed glass evaporating-basin, placed upon an ordinary spring "letter-balance"; the exact quantity used does not matter very much, provided that it is enough, as Kahlbaum's acid, at any rate, is quite free from nitrogen.

In conclusion, I take the opportunity to tender to Professor Pembrey my most sincere thanks for his help and encouragement in this and all my studies, and for the generosity with which he placed all the resources of his laboratory at my disposal.

Summary.

1. The conditions originally suggested by Braunstein in his modification[1] of the Mörner-Sjöqvist process do not give a quantitative yield of the urea-nitrogen.

2. Ignition of the filtrate for 14 hours at 185° with 15 grams of Kahlbaum's crystalline phosphoric acid gives an accurate quantitative result.

[1] In the Mörner-Sjöqvist process, 5 cc. of urine are mixed with 5 cc. of a saturated solution of barium chloride, containing 5 % of barium hydrate; 100 cc. of a mixture of ether and alcohol, in the proportion of 1 : 2, are then added. This is stated, erroneously, to precipitate all the nitrogenous matter, but, as stated above, hippuric acid and creatinine escape. This is the whole point of the present communication.

After leaving the mixture to stand for 24 hrs., the liquor is filtered off, the precipitate is washed with 50 cc. of the alcohol-ether mixture, the washings are added to the filtrate, and a little magnesia is added. The alcohol and ether are driven off at a temperature of 55°, and evaporation is continued until the residue measures 10–15 cc. The nitrogen in this is then estimated by the Kjeldahl method; the result, multiplied by 2·143, is said to equal the amount of urea.

In this paper, it is shown that quantitative estimation of the urea, apart from all other nitrogen, can be obtained, provided that certain experimental conditions are complied with.

XXVI. THE IODOMETRIC ESTIMATION OF SUGARS.

By HILDA MARY JUDD.

From the Department of Plant Physiology and Pathology, Imperial College of Science and Technology, S. Kensington.

(*Received March 6th, 1920.*)

In the course of a series of investigations of the changes that take place in the nature and amount of the carbohydrates of apples during the process of ripening, both in ordinary storage and in cold storage, it has been found that the usual method of estimating fructose by means of the polarimeter is very unsatisfactory. In the first place, the optical activity of apple juice is very low; the sugars present appear to consist mainly of saccharose, glucose, and fructose, the last being in considerable excess, with the result that the juice gives a very small polarimetric reading. Moreover, the juice is always coloured, and is frequently so viscous that it is necessary to dilute it very considerably before it is possible to clear the solution by means of basic lead acetate and alumina cream. The resulting solution seldom contains more than 0·5 % or 1 % of sugars, giving in a 2 decimetre tube a maximum reading of one degree. Obviously the error involved in calculating the proportion of each of the sugars present from such small readings is very large. It becomes clear then that some other method is required.

Several papers dealing with the action of bromine and iodine in alkaline solution on various sugars have been published, some of them quite recently. It has been definitely established that under suitable conditions, both bromine and iodine bring about the oxidation of most sugars, the main product being the corresponding monobasic acid. Bromine in cold aqueous solution slowly oxidises aldoses, and has a still slower action on ketoses. In a mixture of aldose and ketose, however, it is not possible so to control the experiment that only the aldose is attacked.

Bougault [1917] suggests the use of weakly alkaline solutions of iodine. Glucose is then oxidised according to the equation:

$$R.CHO + H_2O + I_2 = R.COOH + 2HI.$$

According to Bougault, fructose when treated in the same way is not appreciably attacked; he admits however that his results are not consistent, and show many discrepancies.

Colin and Lièvin [1918] have attempted to modify and improve Bougault's method. They point out that the degree of alkalinity of the solution may influence the result obtained. While Bougault used a mixture of sodium carbonate and sodium bicarbonate, Colin and Lièvin prefer a solution containing per litre 35 g. of disodium phosphate and 50 cc. of *N* sodium hydroxide.

Romijn [1897] recommends the use of alkaline iodine solutions for the estimation of glucose in presence of fructose. He describes experiments with sodium carbonate, ammonium carbonate, trisodium phosphate, and borax, and prefers the last, as being only very weakly alkaline, and having a less destructive action on the sugar molecule.

The objections to the use of the borax-iodine solution advocated by Romijn are: (1) below 25°, the action is unduly prolonged; (2) at temperatures above 25°, fructose is attacked; (3) in such weakly alkaline solutions, the alkalinity is appreciably reduced by the formation of acid from the sugar.

According to Romijn, all the aldoses behave like glucose, and such di- and tri-saccharides as saccharose and raffinose, which do not reduce Fehling's solution, are also attacked.

Willstätter and Schudel [1918] comment on Romijn's work, and point out that the course of the reaction depends on the amount and on the concentration of the alkali present. There must be sufficient alkali to neutralise the acid formed as a result of the oxidation, but if this amount is exceeded the reaction is not complete. According to Willstätter and Schudel the reaction goes best with decinormal solutions, and in the proportions required by the equation:

$$CH_2OH[CHOH]_4.CHO + I_2 + 3NaOH = CH_2OH[CHOH]_4.COONa + 2NaI + 2H_2O.$$

They state that if these conditions are fulfilled the error with glucose is not more than 1 %. They point out however that in the presence of free alkali the sugars undergo the Lobry de Bruyn transformation.

They find that cane sugar is not acted upon. They also give results with invert sugar, which approximate closely to theory; and they state that under the same conditions fructose is not attacked; no figures however are given for fructose alone.

The following experiments have been carried out in order to determine whether any of the methods mentioned above are applicable to the estimation of the amounts of glucose and fructose present in fruit juices. It has been found, as the result of a large number of determinations carried out with solutions of pure sugars, that neither the method of Colin and Lièvin nor that of Willstätter and Schudel is exact, and that in no case is the sugar oxidised quantitatively to the corresponding monobasic acid; on the other hand, there is always a very definite attack on the fructose. The results obtained with invert sugar are misleading since the low result obtained from the glucose is masked by the partial oxidation of the fructose.

It has further been noticed that the reaction mixture always smells strongly of iodoform, suggesting that some secondary change is taking place, Romijn also draws attention to the fact that a large number of organic substances which give the iodoform reaction are readily oxidised by alkaline iodine solutions.

It seems probable however that the chief source of error is the action of dilute alkalies on sugars, investigated by Lobry de Bruyn and van Ekenstein [1895, 1896, 1897]. Since even such weak alkalies as lead hydroxide bring about this change, it is certain that it would occur in a solution containing a considerable excess of sodium hydroxide. Starting therefore with a solution of pure glucose, part of this sugar would be converted by the alkali into mannose (which is also oxidised by iodine) and part into fructose. Consequently the whole of the sugar present would not be oxidisable by iodine, and the result obtained for the glucose would be too low. Conversely, a solution of fructose would yield a certain proportion of both glucose and mannose, and this probably accounts for the apparent partial oxidation of the fructose.

It may be assumed therefore that the behaviour of the above sugars towards alkaline iodine solutions is in some measure dependent on the Lobry de Bruyn transformation, and that in the presence of iodine some definite equilibrium is reached.

The equilibrium obtained in this way must be a very complex one. According to the observations of Lobry de Bruyn and van Ekenstein, the composition of the almost inactive syrup obtained by the action of dilute alkalies on glucose, mannose, or fructose depends on the nature of the sugar which was used at the start. It appears also from the present series of experiments that the removal of the aldoses present by oxidation does not, as would be expected, tend to convert more ketose into aldose.

It is however possible to utilise the method of Willstätter and Schudel for the analysis of mixtures of glucose and fructose. A large number of experiments show that a given weight of glucose always uses up a definite and constant weight of iodine, although not the amount theoretically required. The same statement holds good for fructose. Neither of these reactions is in any way affected by the presence of other sugars. Experiments with mixtures of glucose and fructose in varying proportions show that the iodine equivalent of the mixture calculated from the two constants in all cases shows very close agreement with that found by experiment. This being the case, it is possible to calculate from the copper reducing power of a mixture of glucose and fructose, and from the amount of iodine used up by the mixture, the proportion of each sugar present.

Experimental details.

The solution of sodium thiosulphate used was carefully standardised by Volhard's method against $N/10$ potassium permanganate. The latter had been standardised in the usual way with ferrous ammonium sulphate and also

against oxalic acid; the values obtained by these two methods showed very close agreement.

The concentration of the sugar solutions used was determined by Bertrand's method, using the same permanganate solution, and the purity of the sugar was also tested by means of the polarimeter. The reaction was carried out in well-stoppered wide-mouthed bottles.

1 cc. *N*/10 iodine contained 0·01293 g. iodine.
1 cc. *N*/10 thiosulphate was equivalent to 0·0128 g. iodine.
(20 cc. iodine = 20·2 cc. thiosulphate.)

A. *Method of Willstätter and Schudel.*

(1) 10 cc. glucose solution (0·978 %).
20 ,, *N*/10 iodine.
30 ,, *N*/10 NaOH.

The mixture was allowed to stand 15–20 minutes at the ordinary temperature, then acidified with dilute sulphuric acid and titrated with thiosulphate.

Titrations: 10·2, 10·1, 10·1, 10·2, 10·15. *Mean*: 10·15.

From this, it follows that 1 g. of glucose requires 1·315 g. of iodine.

(2) 10 cc. fructose solution (1·012 %).
10 ,, *N*/10 iodine.
20 ,, *N*/10 NaOH.

Titrations: 9·3, 9·3, 9·3, 9·3.

From the above it follows that 1 g. of fructose takes up 0·1028 g. of iodine.

B. *Method of Colin and Lièvin.*

Three different solutions were tried, containing varying amounts of alkali, in order to see whether a concentration could be found at which the glucose would be completely oxidised and the fructose remain unaffected.

Phosphate solution I contained 35 g. of sodium phosphate ($Na_2HPO_4\,12\,H_2O$) and 50 cc. of *N* NaOH per litre.

Phosphate solution II contained 35 g. of sodium phosphate and 100 cc. of *N* NaOH per litre.

Phosphate solution III contained 35 g. of sodium phosphate and 25 cc. of *N* NaOH per litre.

The mixtures used were:

10 cc. sugar solution.
20 ,, *N*/10 iodine.
40 ,, phosphate solution.

The mixture was allowed to stand for one hour at the temperature of the laboratory, then acidified with dilute sulphuric acid, and titrated with thiosulphate solution.

The results are given below (Table I). It will be noted that phosphate solutions I and II give results identical with that obtained by Willstätter's method, although solution II contains twice as much sodium hydroxide as

solution I. The third mixture did not contain enough alkali to neutralise the gluconic acid formed. It was repeated, and the results obtained are given under nos. (4), (5) and (6).

(4) Three of the samples were left for two hours to see if the reaction would go further, but the result was negative.

(5) and (6) To each of six other samples was added 10 cc. of $N/10$ sodium hydroxide. Three of them were left for one additional hour, and the rest for two additional hours.

(7) The mixture of glucose and phosphate solution I was heated to 37° for one hour, to see if the reaction would be more complete at a higher temperature. Increase of temperature was however also found to be without influence on the result.

C. *Method of Colin and Lièvin (fructose).*

10 cc. fructose solution (1·012 %).
10 „ $N/10$ iodine.
20 „ phosphate solution.

In all cases the numbers given are the mean of five closely agreeing titrations

Table I.

A. *Willstätter's Method.*

Glucose ...	10·15
Fructose...	9·3

B. *Method of Colin and Lièvin (glucose).*

(1)	Glucose +	phosphate I (1 hr.),		10·15
(2)	„	„	II „	10·15
(3)	„	„	III „	12·62
(4)	„	„	III (2 hrs.),	12·65
(5)	„	„	+ 10 cc. $N/10$ NaOH (3 hrs.),	10·18
(6)	„	„	+ 10 cc. $N/10$ NaOH (4 hrs.),	10·18
(7)	„	„	I at 37° (1 hr.),	10·13

C. *Method of Colin and Lièvin (fructose).*

Fructose +	phosphate I (50 cc. N NaOH per litre),			9·6
„	„	II (100 cc.	„ „),	9·32

The titration corresponding to the complete and quantitative oxidation of glucose to gluconic acid is 9·55. The titration required if fructose is completely unchanged is 10·1. It appears from the above results that glucose is oxidised by alkaline iodine solutions to the extent of about 95 %. The reaction proceeds smoothly, provided that sufficient alkali is present to neutralise the gluconic acid formed. Increasing the amount of alkali does not appear to influence the result, which is also unaffected by heating to 37°.

In the case of fructose, phosphate solution I seems to have caused somewhat less change in the fructose than phosphate solution II, which is the more strongly alkaline, or than Willstätter's reagent, the results obtained with the two last being identical.

There is, however, no advantage in using the method of Colin and Lièvin rather than that of Willstätter and Schudel, since the results are not quantitative in either case. Willstätter's method is preferable on account of its greater simplicity, and also because the time required for the reaction is less.

This method has therefore been adopted, and has been tested with mixtures of glucose and fructose in varying proportions.

The results obtained are given below in tabular form (Table II). The percentage of each sugar present is calculated as follows:

2·369 = weight of CuO equivalent to 1 g. of glucose;
2·369 = „ „ „ „ fructose;
1·315 = „ iodine absorbed by 1 g. of glucose;
0·1028 = „ „ „ „ fructose
Let x = weight of glucose in a given volume V;
„ y = „ fructose „ „
Then $2{\cdot}369x + 2{\cdot}369y$ = wt. of CuO corresponding to V;
$1{\cdot}315x + 0{\cdot}1028y$ = wt. of iodine corresponding to V.

The solutions used were:

$N/10$ iodine; 1 cc. = 0·01293 I;
$N/10$ thiosulphate; 1 cc. = 0·0128 I;
$N/10$ $KMnO_4$; 1 cc. = 0·006892 Cu.

1 cc. glucose = 0·00978 gr. glucose;
1 cc. fructose = 0·01012 gr. fructose.

Table II.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
	cc. glucose solution	cc. fructose solution	cc. $KMnO_4$ (mean)	CuO equivalent of (4)	Titration with $Na_2S_2O_3$ (mean)	Difference from blank	Iodine in (7)	Wt. of glucose		Wt. of fructose	
								taken	calculated	taken	calculated
1	10	10	54·7	0·4719	9·25	10·95	0·1402	0·0978	0·099	0·1012	0·101
2	10	5	40·9	0·3527	9·75	10·45	0·1338	0·0978	0·098	0·0506	0·051
3	10	15	68·5	0·5907	8·95	11·25	0·1440	0·0978	0·098	0·1518	0·151
4	10	20	82·3	0·7098	8·65	11·55	0·1479	0·0978	0·096	0·2024	0·203
5	5	10	41·15	0·3548	4·32	5·78	0·0798	0·0489	0·048	0·1012	0·101
6	15	10	68·25	0·5886	4·33	15·87	0·2032	0·1467	0·147	0·1012	0·102
7	25	10	95·35	0·8222	4·30	26·0	0·3329	0·2445	0·244	0·1012	0·103
8	15	5	54·45	0·4696	4·62	15·58	0·1994	0·1467	0·147	0·0506	0·051

The method has also been tried with a number of other sugars, with results which are of some interest.

The other hexose sugars give results which resemble that obtained with glucose, but are not identical with it. Each sugar appears to have its own characteristic iodine value, and mannose appears to be oxidised with greater difficulty. Of the pentoses tested, arabinose takes up almost the theoretical amount of iodine, while rhamnose is oxidised much less completely. This may

be due to the steric influence of the methyl group. Moreover, the fact that rhamnose does not when oxidised yield the very unstable rhamnonic acid, but a lactone, may influence the reaction.

The results obtained with the disaccharides are remarkable.

The action on saccharose is negligible. In actual experiments the titration differed by 0·1 cc. from the blank, but this is within the limits of experimental error and, further, saccharose almost always contains a trace of reducing sugar.

Maltose behaves quite normally, and the iodine value found by experiment agrees closely with that calculated from the equation:

$$C_{11}H_{21}O_{10}CHO + I_2 = C_{11}H_{21}O_{10}COOH + 2HI.$$

This is also in agreement with the copper-reducing power of maltose.

Lactose, on the other hand, takes up twice as much iodine as is required by the above equation, which fact suggests that the sugar is hydrolysed, and that both the glucose and the galactose molecules are oxidised.

In this connection it is interesting to note that Lobry de Bruyn and van Ekenstein [1895, 1896, 1897] found that when lactose is subjected to prolonged treatment with dilute alkali, it is hydrolysed, as much galactose being produced as in ordinary inversion, together with a little pseudo-tagatose—but no glucose. They assumed that the latter was split off in the form of an anhydride; this does not appear to be the case when iodine is present.

The numerical results are shown in Table III. The solutions used were approximately 2 %, but the actual concentration of the solutions was estimated by Bertrand's method, and checked by means of the polarimeter, except in the case of rhamnose, of which only a small sample was obtainable. In this case however the specimen was very well crystallised, and appeared to be pure, and its copper-reducing power was quite normal.

Table III.

Sugar	Wt. of sugar in 5 cc.	Titration $N/10$ $Na_2S_2O_3$ Mean	Diff. from blank	Iodine equivalent of 1 g. sugar	
				Found	Calculated
Mannose	0·108	11·83	8·17	0·977	1·509
Galactose	0·107	8·25	11·75	1·418	1·509
Arabinose	0·1004	6·65	13·35	1·702	1·69
Rhamnose	0·1087	12·15	7·85	0·9243	1·509
Saccharose	0·100	9·95	0·05	—	—
Maltose	0·0462	14·4	5·6	0·7456	0·75
Lactose	0·104	7·80	12·2	1·502	0·75

Summary.

In dealing with fruit juices, the ordinary method of estimating fructose by means of the polarimeter is unsatisfactory, on account of the low polarimetric readings obtained.

Various iodometric methods for the estimation of aldoses in presence of ketoses have been tested, and none of them has been found to give quantitative results. Experiments with pure sugars show that while the oxidation of glucose is never complete there is always a partial action on fructose. The attempt to find experimental conditions which will ensure the complete oxidation of the glucose while the fructose remains unchanged has been unsuccessful.

It is suggested that the results obtained are due to the behaviour of sugars towards dilute alkalies, which has been investigated by Lobry de Bruyn and van Ekenstein.

In using either the method of Colin and Lièvin or that of Willstätter and Schudel, a definite and constant weight of iodine always reacts with a given weight of glucose. This weight of iodine is not affected by changes in the amount of alkali present, or by the presence of other sugars in the solution. The above statement is also true of fructose. It is therefore possible, using either of the above methods, to calculate from the copper-reducing power of a solution containing glucose and fructose, and from its action on iodine in alkaline solution, the amount of each sugar present in the solution.

The method has also been applied to other sugars. It is shown that each sugar has its own characteristic iodine value. The behaviour of rhamnose is abnormal. The three disaccharides investigated give very different results. Saccharose is not oxidised under the conditions of the experiment. Lactose and maltose are both oxidised, but lactose requires twice as much iodine as maltose.

The work described above has been undertaken in connection with investigations of Cold Storage problems which are being carried out for the Food Investigation Board of the Department of Scientific and Industrial Research.

REFERENCES.

Bougault (1917). *J. Pharm. Chim.* (vii), **16**, 97.
Colin and Lièvin (1918). *Bull. Soc. Chim.* **47**, 403.
Lobry de Bruyn and van Ekenstein (1895). *Rec. Trav. Chim.* **14**, 156, 203.
—— (1896). *Rev. Trav. Chim.* **15**, 92.
—— (1897). *Rec. Trav. Chim.* **16**, 257.
Romijn (1897). *Zeitsch. anal. Chem.* **36**, 18, 349.
Willstätter and Schudel (1918). *Ber.* **51**, 780.

XXVII. DIETETIC EXPERIMENTS WITH FROGS.

By ARTHUR HARDEN and SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute.

(*Received March 8th, 1920.*)

The preliminary experiments of which the results are recorded in the present paper were instituted with the object of ascertaining whether the accessory food factors had any influence on the development and growth of the frog. Both the larval and adult forms of *Rana temporaria* were investigated.

1. *Experiments with Tadpoles.*

These experiments were not entirely satisfactory and are therefore not described in detail. Sets of 6 tadpoles were kept in 200 cc. of boiled tap-water in Erlenmeyer flasks, and were transferred into fresh water every day. The diets were added to the water on five days during the week. They consisted of a basal diet of purified caseinogen (20 %), starch (75 %) and salts (5 %), to which the accessory factors were added in the form of fat-soluble *A*, 4 drops of butter-fat; water-soluble *B*, 0·5 cc. of 1 % marmite; anti-scorbutic *C*, 1 cc. of lemon juice freed from citric acid. For comparison a set of 11 tadpoles was kept in a small aquarium, to which small pieces of raw meat were occasionally added. These control animals throve well, passed through the normal stages and emerged as frogs in the course of 6–9 weeks (with the exception of three which were accidentally lost). On the other hand the experimental animals on the complete diet (Basal + *A* + *B* + *C*) did not succeed nearly so well—only 2 out of 6 reaching the mature state, the others dying on the 15th, 40th, 81st and 155th day in various stages. Similar results were obtained with the diets Basal + *A* and Basal + *A* + *B*, two frogs being produced in the former and one in the latter case.

It is obvious that under these circumstances observations on rates of growth are of no great value and it is proposed to repeat the experiments when a thoroughly satisfactory form of experimental diet has been found. It may however be pointed out that no tadpole reached the mature state which had not had butter-fat in its diet.

The experiments of Emmett and Allen [1919] which were published while our experiments were in progress show that the problem is a very difficult one as the rate of growth and development is greatly affected by the amount and nature of the fat, carbohydrate and protein as well as by the presence or absence of accessory food factors.

2. *Experiments with adult Frogs.*

The frogs were kept in glass basins covered by book muslin and containing a shallow layer of water. They were fed by hand two or three times a week, a bolus of about 0·5–1 g. of the diet being administered. The diets employed were identical with those commonly used for dietetic experiments. The basal mixture consisted of a mixture of caseinogen (20 %), starch (75 %) and salts (5 %), and the accessory factors were added in the form of clarified butter-fat (*A*), autolysed yeast (*B*) and lemon juice freed from citric acid (*C*).

The following diets were used, the composition being in every case adjusted to give a mass of suitable consistency.

	Control	*ABC*	*AB*	*AC*	*BC*	*A*	*B*	*C*
Basal mixture, g.	10	10	10	10	10	10	10	10
Butter-fat, g.	—	1·6	1·85	1·8	—	1·3	—	—
Autolysed yeast, cc.	—	4·7	5	—	4	—	4	—
Anti-scorbutic equivalent to lemon juice, cc.	—	4·7	—	5	4	—	—	4
Olive oil, cc.	3·1	0·24	2·5	1·6	0·6	—	3·1	3·1
Water, cc.	5	—	—	—	—	4	2	2

A disturbing factor was introduced by the fact that some of the animals were attacked by a fungoid disease which caused ulceration of the flesh. The animals which suffered in this way are noted in the tables of results. The frogs were weighed weekly, but it was found that a considerable casual variation occurred. Most of them gained weight during the course of the experiment.

Diet	No.	Orig. wt. g.	Final wt. g.	Duration of experiment days	Result
ABC	5	18·8	26·4	371	Survived
	10	10	26·8	369	„
	11	10·2	14	54	Disease; chloroformed
	27	10	21·4	348	Survived
BC	2	38	47·5	361	„
	4	23·1	35·5	339	Disease; died
	23	10·7	14·5	267	„ „
AC	15	17·3	21	82	Died
	16	12·7	18	74	„
	13*	17	23·2	155	„
	3	17	30·8	42	„
AB	17	15·9	32·1	369	Survived
A	21	17·5	24·5	56	Died
	25	9·8	11·8	37	„
B	19	14·6	20	70	„
	20	15	27·6	369	Survived
C	22*	18·2	25·3	91	Died
	26*	13·9	20·6	73	„
Control	6	10·2	15·1	52	„
	7	27·1	31	112	Disease; died
	12*	9·2	19·1	26	Died
	9*	13·2	12·8	22	„
	28	12·4	16·8	111	„

In the cases marked * (Nos. 13, 22, 26, 12 and 9) the animals took up a characteristic attitude before death, which is often adopted by moribund frogs, with the hind legs extended and the fore part of the body raised on the fore limbs.

In several cases (Nos. 6, 15, 16, 22, 25, 26) a nerve was removed and submitted to Miss E. M. Tozer to whom we are indebted for their microscopic examination. In only one case (No. 6) was any abnormality observable. The nerve (the sciatic) had very few nodes and the sheath had an eroded appearance.

In spite of the small number of animals on some of the diets, the following conclusions appear to be justified:

1. All the 7 frogs deprived of both *A* and *B* died within four months. Of these animals 4 showed the "extended legs" symptom; one suffered from the fungus disease.

2. Of the 5 animals receiving both *A* and *B*, with or without *C*, only one died during a whole year and this one was attacked by the fungus.

3. Of the 6 animals receiving *A* (with or without *C*) but not *B*, all died within 155 days, one showing the extended legs symptom.

4. Of the 5 animals receiving *B* (with or without *C*) but not *A*, 4 survived for 250 days. Two of these subsequently died (at 267 and 339 days), both having shown signs of external haemorrhage.

5. Considering the whole set of animals, 11 received *A* in their diet and 12 did not. After 6 months 7 of those receiving *A* and 9 of those not receiving *A* were dead. On the other hand 10 received *B* in their diet and 13 did not. After 6 months all those not receiving *B* and only 2 of those receiving *B* were dead.

This affords clear evidence that *B* is necessary for the continued existence of adult frogs. On the other hand the effect of absence of *A* does not show itself definitely within 6 months. The absence of *C* in these experiments did not produce any definite effect.

In order to obtain definite confirmation of this result 6 frogs were kept on a complete *ABC* diet and 6 on an *AC* diet, with the following results:

Diet	No.	Orig. wt.	Final wt.	Duration of experiment days	Result
ABC	29	26·9	28·7	273	Disease; survived
„	30	26·9	28·3	273	Survived
„	31	24	20·8	154	Disease; died
„	32	30·2	30·9	273	Survived
„	33	23·2	23·9	273	„
„	34	21·2	21·4	272	Disease; survived
AC	35	26·7	28·5	255	Died
„	36	26·7	26·5	241	„
„	37	27·3	27·3	229	„
„	38	24·7	22·2	146	Disease; chloroformed
„	39	20·7	19·8	149	Died
„	40	18·6	19·8	219	„

In this set, not considering two frogs (31 and 38), one on each diet, which died while suffering from the fungus, we have 5 on *ABC* diet, all of which survived 272 days, and 5 on *AC* diet, all of which succumbed within 255 days, the actual duration of life being 255, 241, 229, 149 and 219 days respectively.

Characteristic symptoms were only noticed in two cases. No. 39 adopted the "extended legs" position 3 days before death. No. 40 became rigid after being fed on the 14th July, gradually recovered, but had a second similar attack, preceded by a series of convulsions on the 17th July, and was found dead on the 18th. The sciatic nerve was examined in both these cases with the result that the nerve of No. 39 was normal, whereas that of No. 40 contained several degenerating fibres.

These results show that the addition of autolysed yeast juice to the *AC* diet employed in these experiments greatly prolongs the life of the frog. This is most probably due to the presence of the water-soluble *B* factor, but it must be remembered that the autolysed yeast juice also contains a considerable proportion of easily absorbable nitrogen which may also have some effect. Further experiments in which a basal diet richer in protein is being employed are however yielding precisely similar results to those described above.

Part of the expenses of the research was defrayed from a grant made by the Medical Research Council, to whom our thanks are due.

Summary.

A series of feeding experiments with synthetic diets indicates that the water-soluble *B* factor is necessary for the continued life of the adult frog.

REFERENCE.

Emmett and Allen (1919). *J. Biol. Chem.* **38**, 325.